

REMARKS

Claims 1, 5, 15, 41 – 43, 45 and 59 - 70 were pending in this application. Claims 59 – 66 were withdrawn from consideration. Claims 2, 3, 4, 6 – 14, 16 – 40, 46 – 58 and 67 - 68 were cancelled. Claims 1, 41, 42 and 43 have been amended. No new claims were added.

Any cancellation of the claims should in no way be construed as acquiescence to any of the Examiner's rejections and was done solely to expedite the prosecution of the application. Applicant reserves the right to pursue the claims as originally filed in this or a separate application(s).

Oath/ Declaration

The Examiner has indicated that the Oath/Declaration is defective and a new Oath/Declaration in compliance with 37 CFR 1.67 identifying the application by application number and filing date is required. The Examiner indicates that the second inventor has made non-initialed and/or non-dated alterations to the oath or declaration.

Applicants submit a new oath/declaration in compliance with 37 CFR 1.67(a) under separate cover.

Drawings

The Examiner indicates that the drawings filed on August 20, 2004 are objected to because it appears that some of the Figures are color photographs where only black and white Replacement Figures have been submitted.

Applicants refer to the Standards for drawings set forth at 35 CFR §1.84 which states that "(o)n rare occasions, color drawings may be necessary as the only practical medium by which to disclose the subject matter sought to be patented in a utility or design patent application or the subject matter of a statutory invention registration." Applicants submit that in the present application, black and white Replacement Figures are sufficient and color Figures are not necessary. Applicants have amended the specification accordingly.

Applicants respectfully request that the rejection be withdrawn.

Nucleotide Sequence Disclosures

The Examiner indicates that the application contains sequences which fall under the purview of 37 CFR 1.821 through 1.825 as requiring SEQ ID NOs, but which are not so identified. The Examiner indicates that Applicant must fully comply with the requirements of 37 CFR 1.821 – 1.825 in order for any response to this action to be fully responsive.

Applicants submit a sequence listing in compliance with 37 CFR 1.821 through 1.825 and respectfully request that the rejection be withdrawn.

Specification

The Examiner has acknowledged Applicant's reference to priority in the first sentence of the specification.

The Examiner has objected to the disclosure for containing embedded hyperlinks or other forms of browser executable code that are impermissible and must be deleted. Applicants have made the appropriate corrections to remove embedded hyperlinks and respectfully request that the objection be withdrawn.

Claim Objections

The Examiner has objected to claim 41 for minor informalities. Applicants have amended the claim and respectfully request that the objection be withdrawn.

Claim Rejections 35 USC 112, First Paragraph

Claims 1, 5, 15, 41 – 43, 45 and 67 – 70 have been rejected under 35 USC 112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner argues that “the specification, while being enabling for an in vitro method for inhibiting replication or transcription of a nucleic acid molecule indicative of disease state, or an in vitro

method for selectively treating cells comprising an infectious disease organism, does not reasonably provide enablement for an in vivo method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, or an in vivo method for selectively treating cells comprising an infectious disease organism. (Office Action, p.7). Applicants respectfully disagree.

Claim 1 recites a method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, the method comprising targeting the nucleic acid molecule with an oligonucleotide; and, binding of the oligonucleotide to the target nucleic acid molecule; wherein the oligonucleotide comprises a backbone nucleic acid sequence, and two arm nucleic acid sequences, and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule, wherein the oligonucleotide backbone has one or more mismatches with the target nucleic acid sequence, thereby inhibiting transcription of the target nucleic acid molecule.

Claim 41 recites a method for selectively treating cells comprising an infectious disease organism, comprising administering to the cells an oligonucleotide sequence that is complementary to a target nucleic acid molecule of an infectious disease organism, the cells comprising an oligonucleotide sequence of an infectious disease organism; wherein the oligonucleotide comprises a backbone nucleic acid sequence and two arm nucleic acid sequences, and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule; wherein the oligonucleotide backbone has one or more mismatches with the target nucleic acid sequence, thereby inhibiting transcription of the target nucleic acid molecule.

The MPEP states that the determination that “undue experimentation” would have been needed to make and use the claimed invention is not a single, simple factual determination. Rather, it is a conclusion reached by weighing a combination of factual considerations: the breadth of the claims, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, the amount of direction or guidance presented, the presence or absence of working examples, and the quantity of experimentation necessary. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Accordingly, the Examiner has considered the following factors in his rejection:

The nature of the invention and the breadth of the claims

According to the MPEP at 2164. 05(a), "whether the specification would have been enabling as of the filing date involves consideration of the nature of the invention, the state of the prior art, and the level of skill in the art. The initial inquiry is into the nature of the invention, i.e., the subject matter to which the claimed invention pertains. The nature of the invention becomes the backdrop to determine the state of the art and the level of skill possessed by one skilled in the art."

As to the nature of the invention, the Examiner alleges only that "the instant claims are drawn to a method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, or a method for selectively treating cells comprising an infectious disease organism...(and) the broadness of the methods recited in the claims implies in vivo applicability for enablement purposes." (Office Action, p.8). The Examiner argues that "(t)he nature of the invention, therefore, requires the knowledge of using oligonucleotide molecules that can be delivered into cells in a subject (in vivo) such that transcription of the target nucleic acid is inhibited." (Office Action, p.8). Applicants disagree.

Applicants submit that the nature of the invention, the state of the prior art, and the level of skill in the art were sufficiently developed at the time of filing to enable one of skill in the art to perform the invention as claimed. Applicants direct the Examiner to page 2, beginning at line 8, for example, where Applicants list some of the references that teach molecular based approaches to target cells:

While existing approaches to target cells based on their genotype is limited, some molecular based approaches have been developed. These include antisense RNA [(Izant, J. G. & Weintraub, H. Science 229, 345-52. (1985); Detrick, B. et al. Invest. Ophthalmol. Vis. Sci. 42, 163-9. (2001); Miller, P. S., Cassidy, R. A., Hamma, T. & Kondo, N. S. Pharmacol. Ther. 85, 159-63. (2000)], triplex DNA [(Blume, S. W., Gee, J. E., Shrestha, K. & Miller, D. M. Nucleic Acids Res 20, 1777-84. (1992); Chan, P. P. & Glazer, P. M. J. Mol. Med. 75, 267-82. (1997); Cassidy, R. A., Kondo, N. S. & Miller, P. S. Biochemistry 39, 8683-91. (2000)], ribozymes [(Beaudry, A. A. & Joyce, G. F. Science 257, 635-41. (1992); Joyce, G. F. Science 289, 401-2. (2000)], "suicide" gene therapy [(Shimura, H. et al.

Cancer Res. 61, 3640-6. (2001); Black, M. E., Kokoris, M. S. & Sabo, P. Cancer Res. 61, 3022-6. (2001)], and inhibitory RNA [(Elbashir, S. M. et al. Nature 411, 494-8 (2001); Brummelkamp, T. R., Bernards, R. & Agami, R. Science 296, 550-3 (2002)].

Accordingly, Applicants argue that given the nature of the invention, the claims are enabled as written.

Working Examples and Guidance Provided

According to the MPEP at 2164.02, “compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, does not turn on whether an example is disclosed.” Moreover, “an applicant need not have actually reduced the invention to practice prior to filing. In *Gould v. Quigg*, 822 F.2d 1074, 1078, 3 USPQ 2d 1302, 1304 (Fed. Cir. 1987). The Court held that “the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it.” 822 F.2d at 1078, 3 USPQ2d at 1304 (quoting *In re Chilowsky*, 229 F.2d 457, 461, 108 USPQ 321, 325 (CCPA 1956)). “The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. In re: *Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970).”

The Examiner alleges that “(t)he specification as filed does not provide sufficient guidance or appropriate examples that would enable a skilled artisan to use the claimed methods in *in vivo* environments. (Office Action, p.8). The Examiner argues further that “a person skilled in the art would recognize that predicting the efficacy of a compound...based solely on its performance *in vitro* is unpredictable.” (Office Action, p.8 - 9). Applicants disagree.

The Examiner’s argument is misplaced. As stated by the MPEP above, the specification does not need to contain an example if the invention is disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. Applicant does not need to demonstrate the method in an *in vivo* environment, as argued by the Examiner, to enable the invention as claimed. Applicant has provided ample data in the instant disclosure supporting a method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state or a method for selectively treating cells comprising an infectious disease organism, as claimed. Applicants point out Example 2, which shows that the

gene targeting antigene locks bind in a sequence specific manner and can be "locked." FIG. 2A-2D shows that the antigene lock structures bind specifically to their targets, and in the presence of DNA ligase, inhibit DNA synthesis in-vitro. Example 3 shows that antigene locks inhibit in-vitro DNA synthesis. Since the antigene locks bind their gene target in a sequence specific manner in vitro and inhibit in vitro DNA synthesis, Example 4 tests these locks for their binding to a gene target inside a cell. In Example 4, a gene target was selected that was present in cells at low copy number, but would be reliably replicated and transmitted to daughter cells under normal conditions. The DNA target was selected for ease of monitoring its presence or absence, and was non-essential for cell survival so that its loss could be detected in surviving cells. Applicants found that with both sequence specific locks, white colonies were produced on these plates at frequencies above those seen with the plasmid alone. When the control antigene locks were transformed into the same 8036/+6 cells, very few white colonies were produced. The frequencies that white colonies were produced with the antigene locks were significantly higher than their respective controls ($p=0.01$ and $p=0.03$). In Example 8 Applicants show that Alu and HPV-E7 antigene locks kill human cervical cancer cells. Figure 6, that shows gene specific antigene locks can kill human cervical cancer cells. FIG. 6A is a schematic illustration showing the position of the antigene locks on their targets on the alu repeat and the HPV-16 E7 oncogene. FIG. 6B shows that a gene specific alu antigene lock specifically kills human cervical cancer cells. FIG. 6C is a bar graph showing gene specific E7 antigene lock selectively kills human cervical cancer cells, CaSki and C33A/E7 that contain the E7 gene target.

Accordingly, Applicants argue that given the working examples and guidance provided, the claims are enabled as written.

State of the Art

According to the MPEP at 2164. 05(a), "(t)he state of the prior art is what one skilled in the art would have known, at the time the application was filed, about the subject matter to which the claimed invention pertains (and) the state of the art for a given technology is not static in time. It is entirely possible that a disclosure filed on January 2, 1990, would not have been enabled. However, if the same disclosure had been filed on January 2, 1996, it might have enabled the claims. Therefore, the state of the prior art must be evaluated for each application based on its filing date."

The Examiner alleges that "the claimed invention is a class of invention which the CAFC has characterized as 'the unpredictable arts such as chemistry and biology' (and) (t)he claims encompass *in vivo* methods for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, or *in vivo* methods for selectively treating cells...however the specification only shows that such methods are carried out *in vitro*." (Office Action, p.9).

Applicants disagree. The instant application provides detail regarding the feasibility of the claimed methods at the time of filing. For example, Applicants direct the Examiner to the references and Examples cited above.

At page 22 of the specification, Applicants describe "gene delivery" or "gene transfer" as referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgenes") into a host cell, irrespective of the method used for the introduction. Applicants describe a number of methods known in the art that have been used for gene delivery *in vivo*:

Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, e.g., viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of naked polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (e.g., a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein. (page 22 – 23)

As pointed out by Applicants above, electroporation and gene gun delivery were both known and used at the time of filing to deliver genes *in vivo*. For example, Rakhmilevich et al. (Proc Natl Acad Sci U S A. 1996 June 25; 93(13): 6291–6296; enclosed herewith) in a 1996 publication, describe particle-mediated (gene gun) *in vivo* delivery of the murine interleukin 12 (IL-12) gene in an expression plasmid. Sun et al. (Proc Natl Acad Sci U S A. March 28, 1995 vol. 92 no. 7 2889-2893; enclosed herewith) in a 1995 publication, describe an approach to tumor immunotherapy utilizing direct transfection of cytokine genes into tumor-bearing animals by particle-mediated gene transfer. Nishizaki et al. (Ann Thorac Surg 2000;70:1332-1337;

enclosed herewith) in a 2000 publication, demonstrate gene gun-mediated transfer into a beating heart. Clearly, the claimed methods were feasible and enabled at the time of filing

Applicants have provided ample evidence that the methods as instantly claimed are enabled by the instant disclosure.

Taken together, the teachings of the specification and knowledge of one of skill in the art enables one of skill in the art to practice the full scope of the claimed invention without having to resort to undue experimentation. Applicants accordingly request that the rejection be reconsidered and withdrawn.

Claim Rejections 35 USC 103(a)

Claims 1, 5 and 15 have been rejected under 35 USC 103(a) as being unpatentable over Gibson et al. (Clinical Cancer Research, 2000) in view of Escude et al. (PNAS, 1999). Applicants respectfully disagree.

Claim 1 recites a method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, the method comprising targeting the nucleic acid molecule with an oligonucleotide; and, binding of the oligonucleotide to the target nucleic acid molecule; wherein the oligonucleotide comprises a backbone nucleic acid sequence, and two arm nucleic acid sequences, and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule, wherein the oligonucleotide backbone has one or more mismatches with the target nucleic acid sequence, thereby inhibiting transcription of the target nucleic acid molecule.

Accordingly, the present invention provides methods for selective killing of cells based on their genotype. The claimed methods utilize oligonucleotides that bind specifically to their gene targets, intertwine with both strands of the target DNA and thereby inhibit DNA synthesis. When transformed into a mixed population of cells, where only one cell type possesses the target, the oligonucleotides of the invention selectively kill only the target bearing cell population. The oligonucleotides taught in the present invention are considerably different from other molecular approaches taken in the prior art.

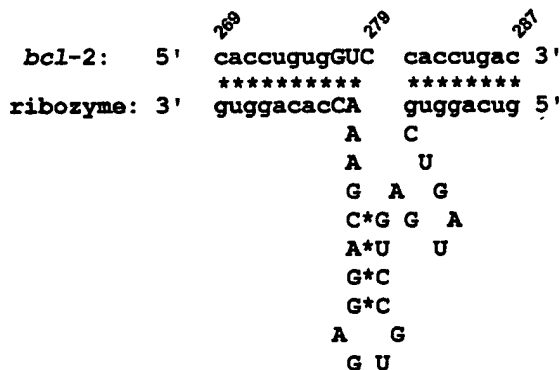
The Examiner argues that the Gibson reference teaches “a method for inhibiting transcription in a mammalian cell in vitro comprising administering an anti-bcl-2 ribozyme.”

(Office Action p.15). The Examiner contends that the Gibson reference teaches that “bcl-2 is commonly over-expressed in non-oral cancers, such as follicular lymphomas, and this satisfies that bcl-2 is a nucleic acid molecule indicative of a disease state.” (Office Action, p.15). The Examiner admits that “Gibson et al do not teach an oligonucleotide comprising a backbone nucleic acid sequence, and two arm nucleic acid sequence, and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule.” (Office Action, p.15).

The Gibson reference is directed to the induction of apoptosis in oral cancer cells by an anti-bcl-2 ribozyme delivered by adenovirus. The present invention provides methods for selective killing of cells based on genotype using oligonucleotides that bind specifically to gene targets. The ribozymes taught by the Gibson reference are structurally and functionally different from the oligonucleotides that are taught and claimed in the present application. Ribozymes are RNA molecules that are capable of catalyzing RNA cleavage in a sequence-specific manner. As taught in the present specification, for example at page 39, beginning at line 21:

Ribozymes, linear oligonucleotides with a loop structure, are catalytic nucleic acids, which are designed to inactivate specific mRNA. Antigen locks do not possess catalytic properties and are very different in structure. Ribozymes are dependent on magnesium, antigen locks are not.

Applicants direct the Examiner to Figure 1, below, which shows the anti-bcl-2 ribozyme and its target. Ribozymes are antisense RNA molecules that catalytically cleave specific target RNA, **leading to degradation**. The ribozyme taught by the Gibson reference cleaves the transcript of the bcl-2 gene between nucleotides 279 and 280. (p.213).



Not only is the method of but the present invention is directed to inhibiting transcription of the target nucleic acid molecule while ribozyme technology leads to degradation of target RNA.

Nowhere does the Gibson reference teach or suggest a method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state where the method comprises targeting the nucleic acid molecule with an oligonucleotide and binding of the oligonucleotide to the target nucleic acid molecule, wherein the oligonucleotide comprises a backbone nucleic acid sequence, and two arm nucleic acid sequences, **and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule, thereby inhibiting transcription of the target nucleic acid molecule**, as presently claimed.

The Escude reference does not cure the defects of the Gibson reference. Neither reference, taken alone or in combination, teaches or suggests the invention as claimed.

The Escude reference teaches the design of an oligonucleotide, in particular oligonucleotides that target oligopurine-oligopyrimidine sequence from the promoter of the mouse androgen receptor, that winds around the DNA double helix and can then be circularized. (p.10604). Referring to Figure 2, Escude et al teach “an 89-mer oligonucleotide that contain(s) a central triple helix forming sequence connected by two T19 linkers to sequences that could form 10 base pairs each with a 20-mer oligonucleotide.” (p.10604).

The present invention teaches **backbone nucleic acid sequences that are complementary to one strand of the target nucleic acid molecule and arms that are complementary to the other strand of the target nucleic acid molecule, where the oligonucleotide backbone has one or more mismatches with the target nucleic acid sequence**. Escude describes an enzymatic method that allows the assembly of an artificial sliding clamp directly on a circular plasmidic DNA through the use of a triple-helical complex. (p.10603). Applicants refer to Examiner to Figure 1, which is a schematic diagram of the locking technique. Figure 1 shows

(t)he central part of a linear oligonucleotide (yellow) binds to the major groove of a specific sequence within a plasmid (red and blue) to form a triple-helical complex. Then, its 5' and 3' ends hybridize adjacent to each other to a template

oligonucleotide (green) and are joined together by T4 DNA ligase (white) to form a circular oligonucleotide to form a circular oligonucleotide catenated to the plasmid DNA.

Figure 2 shows the triplex site formed by the 89-mer by binding to a target sequence in the pAR plasmid. Escude does not teach nucleic acid sequences that are complementary to one strand of the target nucleic acid molecule and arms that are complementary to the other strand of the target nucleic acid molecule, where the oligonucleotide backbone has one or more mismatches with the target nucleic acid sequence.

The Examiner argues that “one of ordinary skill in the art would have been motivated to devise a method for inhibiting replication or transcription of a nucleic acid molecule indicative of a disease state, the method comprising targeting the nucleic acid molecule with an oligonucleotide since Gibson et al. taught that such a method could cause cancer cells to lose their malignant behavior.” (Office Action, p.16). The Examiner argues that “(o)ne of ordinary skill in the art would have been motivated to substitute the anti-bcl ribozyme taught by Gibson et al. with an oligonucleotide comprising a backbone nucleic acid sequence, and two arm nucleic acid sequence” as taught by the Escude reference. (Office Action, p. 16).

One of ordinary skill in the art would not be motivated to use the oligonucleotide taught by the Escude reference in the method of Gibson, or in any method comprising targeting any nucleic acid molecule with an oligonucleotide with any reasonable expectation of success. Not only are the oligonucleotides taught by Escude structurally different from the claimed invention, but the ribozyme technology, where RNA is degraded, is completely different from any use taught by the Escude reference. Escude suggest use of the padlock oligonucleotides for cell transfection procedures, plasmid purification, gene transfer (p.10606).

Accordingly, Applicants respectfully request that the foregoing rejection be withdrawn.

Claims 41 – 43 and 45 have been rejected under 35 USC 103(a) as being unpatentable over Zhang et al. (FEBS Letters, 1999) in view of Escude et al. (PNAS, 1999). Applicants respectfully disagree.

Claim 41 recites a method for selectively treating cells comprising an infectious disease organism, comprising administering to the cells an oligonucleotide sequence that is complementary to a target nucleic acid molecule of an infectious disease organism, the cells comprising an oligonucleotide sequence of an infectious disease organism; wherein the

oligonucleotide comprises a backbone nucleic acid sequence and two arm nucleic acid sequences, and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule; wherein the oligonucleotide backbone has one or more mismatches with the target nucleic acid sequence, thereby inhibiting transcription of the target nucleic acid molecule.

The Examiner argues that “Zhang et al. teach the in vitro cleavage of an anti-HIV-1 DNAzyme, DzV3-9 (and)...that the anti-HIV1 DNAzyme was administered, detected and stabilized in mammalian cells.” (Office Action, p.18). The Examiner argues that Zhang “also teach that mammalian cells were infected with a virus and virus replication was inhibited upon administration of DzV3-9.” (Office Action, p.18 -19). The Examiner admits that the Zhang et al. “do not teach an oligonucleotide comprising a backbone nucleic acid sequence, and two arm nucleic acid sequence, wherein the oligonucleotide comprises a backbone nucleic acid sequence and two arm nucleic acid sequences, and wherein the backbone nucleic acid sequence is complementary to one strand of the target nucleic acid molecule and the arms are complementary to the other strand of the target nucleic acid molecule.

The Escude reference does not cure the defects of the Zhang reference. Neither reference, taken alone or in combination, teaches or suggests the invention as claimed.

Accordingly, Applicants respectfully request that the foregoing rejection be withdrawn.

CONCLUSION

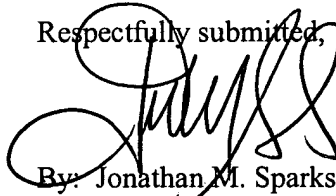
In view of the above remarks, Applicants respectfully request withdrawal of the rejection and allowance of the claims.

A two month extension of time is requested.

If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney of record. The Director is hereby authorized to charge any credits or deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105.

Dated: March 30, 2009

Respectfully submitted,



By: Jonathan M. Sparks, Ph.D.

Registration No.: 53,624

EDWARDS ANGELL PALMER & DODGE
LLP

P.O. Box 55874

Boston, Massachusetts 02205

(617) 517-5543

Attorneys/Agents For Applicant

Gene gun-mediated skin transfection with interleukin 12 gene results in regression of established primary and metastatic murine tumors

ALEXANDER L. RAKHMILEVICH*, JOEL TURNER*, MAURA J. FORD*, DENNIS MCCABE*, WENN H. SUN†, PAUL M. SONDEL‡, KAREN GROTA*, AND NING-SUN YANG*§

*Cancer Gene Therapy, Auragen Inc., Middleton, WI 53562; †Department of Dermatology, Children's Memorial Hospital at Northwestern University, Chicago, IL 60614; and ‡Departments of Pediatrics, Human Oncology, and Genetics, University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792

Communicated by C.-C. Tan, Fudan University, Shanghai, People's Republic of China, March 15, 1996 (received for review December 25, 1995)

ABSTRACT Particle-mediated (gene gun) *in vivo* delivery of the murine interleukin 12 (IL-12) gene in an expression plasmid was evaluated for antitumor activity. Transfer of IL-12 cDNA into epidermal cells overlying an implanted intradermal tumor resulted in detectable levels (266.0 ± 27.8 pg) of the transgenic protein at the skin tissue treatment site. Despite these low levels of transgenic IL-12, complete regression of established tumors (0.4–0.8 cm in diameter) was achieved in mice bearing Renca, MethA, SA-1, or L5178Y syngeneic tumors. Only one to four treatments with IL-12 cDNA-coated particles, starting on day 7 after tumor cell implantation, were required to achieve complete tumor regression. This antitumor effect was CD8⁺ T cell-dependent and led to the generation of tumor-specific immunological memory. By using a metastatic P815 tumor model, we further showed that a delivery of IL-12 cDNA into the skin overlying an advanced intradermal tumor, followed by tumor excision and three additional IL-12 gene transfections, could significantly inhibit systemic metastases, resulting in extended survival of test mice. These results suggest that gene gun-mediated *in vivo* delivery of IL-12 cDNA should be further developed for potential clinical testing as an approach for human cancer gene therapy.

Interleukin 12 (IL-12), a bimolecular glycoprotein consisting of a 35- and a 40-kDa subunit, was originally identified as a factor that stimulates natural killer cells (1, 2) and promotes maturation of cytotoxic T lymphocytes (CTL) (3, 4). It has recently been demonstrated that local or systemic treatment with recombinant (r) IL-12 protein mediates profound antitumor effects *in vivo*, causing regression of established subcutaneous tumors and tumor metastases (5, 6). However, systemic administration of rIL-12 caused dose-dependent toxicity in mice (7) and in human trials (8). Thus, a delivery mechanism that can provide relatively low levels of IL-12 at the target tissue might be advantageous in that it could generate an antitumor effect without causing systemic toxicity. Indeed, as cancer gene therapy has evolved, recent studies have produced encouraging results, showing that murine fibroblasts (9) or tumor cells (10) transduced *in vitro* with the IL-12 gene using a retroviral vector were able to induce antitumor immune responses. These data suggest that peritumoral IL-12 delivery may be as efficacious as systemic administration and avoid many undesirable side effects.

The particle-mediated method for gene delivery by gene gun utilizes a shock wave to accelerate DNA-coated gold particles into target cells or tissues. At submicrogram quantities of DNA per dose for *in vitro* or *in vivo* gene transfer, the gene gun can deliver thousands of DNA copies intracellularly into test

tissues, resulting in high level transgene expression (11). As this method is cell surface receptor-independent, it can successfully deliver genes into a wide spectrum of mammalian cell types (12, 13). We have recently demonstrated that a particle-mediated, *in vivo* cytokine gene therapy reduces tumor growth in mice (14). Treatments with interferon- γ and tumor necrosis factor α shortly after the implantation of tumor cells inhibited tumor growth and prolonged the survival of tumor-bearing mice. To more closely approximate clinical situations, and to take advantage of the findings that IL-12 more effectively stimulates activated than naive T cells (15, 16), we evaluated the effect of IL-12 gene therapy on the growth of established tumors. In this study, we utilized the gene gun technology for *in vivo* IL-12 cDNA delivery into the skin overlying the implanted, established tumor tissues.

MATERIALS AND METHODS

Mice. BALB/c, C57BL/6, DBA/2, and A/J female mice between 8 and 12 weeks of age were obtained from Harlan-Sparague-Dawley or Taconic Farms. All animal experiments were conducted in accordance with principles stated in ref. 17.

Murine Tumor Models. Six established mouse tumor cell lines were employed in this study, namely Renca carcinoma, MethA sarcoma (both syngeneic in BALB/c mice), L5178Y lymphoma, P815 mastocytoma (both syngeneic in DBA/2 mice), SA-1 sarcoma, and B16 melanoma (syngeneic in A/J and C57BL/6 mice, respectively). Tumor cell cultures were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and gentamicin at 50 μ g/ml. MethA sarcoma, SA-1 sarcoma, P815 mastocytoma, and L5178Y lymphoma were grown as ascites in syngeneic mice for 1 week before injecting intradermally (i.d.). Mice were shaved in the abdominal area and injected i.d. with 1×10^6 (or 1×10^5 , in the case of B16 tumor) tumor cells in 50 μ l phosphate-buffered saline (PBS). Tumor growth was monitored two to three times a week by measuring two perpendicular tumor diameters using calipers.

IL-12 Gene Expression Vector. We constructed a plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of murine (m) IL-12, linked tandemly in the same direction and each driven by its own cytomegalovirus (CMV) i/e promoter/enhancer, a simian virus 40 (SV40) sd/sa intron sequence, and a bovine growth hormone polyadenylation sequence. The murine 35- and 40-kDa IL-12 subunit cDNA clones were isolated from mouse lymphocyte cDNA libraries by PCR cloning. The PUC19 plasmid backbone was derived from a bluescript SK(+) vector with an ampicillin-resistance gene (see Fig. 1A). A control vector containing a luciferase

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Abbreviations: IL-12, interleukin 12; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody; r, recombinant; m, murine; CMV, cytomegalovirus; SV40, simian virus 40; Luc, luciferase.

§To whom reprint requests should be addressed.

(Luc) cDNA expression plasmid containing the CMV promoter was constructed as described by Cheng *et al.* (12).

In Vivo and in Vitro Gene Transfer. The experiments utilized a helium-pulse Accell (gene gun) device that was designed by D. McCabe (Agracetus, Inc). Plasmid DNA was precipitated onto 2 μ m gold particles. Particles were suspended in a solution of 0.1 mg of polyvinyl pyrrolidone per ml in absolute ethanol. This DNA/gold/particle preparation was coated onto the inner surface of a Tefzel tubing by using a tube loader (Agracetus), and the tubing was cut into 0.5-inch segments to result in delivery of 0.5 mg gold and 1.25 μ g plasmid DNA per transfection. For tumor therapy, mouse skin overlying and surrounding the target tumor was transfected *in vivo* with IL-12 or Luc cDNA expression vectors starting from day 7 after i.d. implantation of 1×10^6 of five different types of tumor cells, except for B16 tumor, which was implanted at 10^5 cells. Each treatment consisted of four transfections (5 μ g plasmid DNA/treatment) with a 300 psi helium gas pulse. One transfection was directly over the tumor site, and three additional treatments were evenly spaced around the circumference of the tumor in a triangle pattern. The *in vitro* particle bombardment gene transfer was performed as described (18).

IL-12 Bioassay. For determining transgenic IL-12 expression following *in vivo* gene transfer, blood was obtained by cardiac puncture, and skin tissue samples containing four transfection sites were collected in 0.5 ml of general extraction buffer, thoroughly minced with scissors, and sonicated before collecting the supernatant. The level of transgenic IL-12 protein was determined by a cell proliferation bioassay by using murine Con A-activated splenocytes as described (19). Briefly, spleen cells (5×10^6 /ml) from naive BALB/c mice were stimulated with Con A (5 μ g/ml) for 4 days at 37°C. Serial dilutions of the test samples (cell culture supernatants, serum, or skin tissue homogenates) were incubated with the activated spleen cells (2×10^4 cells/well) for 48 h, and the level of cell proliferation was measured by [3 H]thymidine incorporation. Serially diluted recombinant murine IL-12 (R & D Biosystems)

was used as a standard. Anti-mIL-12 monoclonal antibody (mAb) (kindly provided by M. Gately, Hoffman-La Roche) was used to ensure that the bioactivity of the samples was due to IL-12. The sensitivity of this assay was about 10 pg/ml for rIL-12 standard protein and cell culture samples, and about 100 pg/ml for serum and skin tissue extracts.

Immunohistochemistry. The *in vivo* transfected skin tissues were sectioned in a cryostat (8 mm), placed on silanated slides and allowed to air dry. Test tissues were then fixed with acetone at 4°C for 10 min, air dried, washed in PBS for 10 min, and incubated with the anti-IL12 mAb (10 mg/ml) for 60 min at room temperature. Reacted tissues were rinsed two times in PBS and incubated with a biotinylated secondary antibody (rabbit anti-rat IgG; Vector Laboratories) for 60 min at room temperature. After rinsing with PBS, localization of the antibody binding was visualized with peroxidase staining and developed with metal enhanced 3,3'-diaminobenzidine- H_2O_2 . Sections were rinsed in PBS and counterstained with hematoxylin, dehydrated in ethanol, treated with xylene, and mounted with permount.

IL-12 Gene Therapy of Spontaneous Metastasis. A spontaneous metastasis model using weakly immunogenic P815 tumor has been described (20). DBA/2 mice were injected i.d. with 1×10^6 P815 cells. The skin overlying and surrounding the tumor was transfected with IL-12 cDNA or Luc cDNA on days 12 and 14 of tumor growth. Surgical excision of the tumor was performed on day 15 of tumor growth, and additional transfections of the skin on both sides of abdomen were performed on days 16, 18, and 20. Survival of the mice was followed.

Generation of Cytotoxic T Lymphocytes and Cytotoxic Assay. Tumor-specific CTL were generated *in vitro* as described (21). Briefly, spleen cells (5×10^6), derived from BALB/c mice that had rejected Renca tumors due to IL-12 gene therapy and had remained tumor-free for 2 months, or from age-matched naive mice, were cocultured with 5×10^4 mytomicin C-treated Renca cells in 24-well culture plates in complete RPMI 1640 media. After culturing for 5 days *in vitro*,

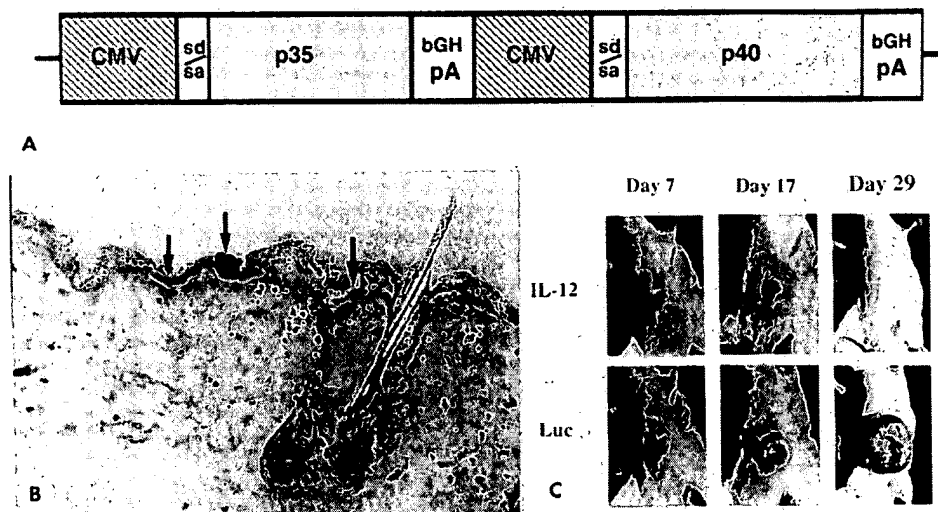


FIG. 1. *In vivo* transfer of IL-12 cDNA expression plasmid into mouse skin leads to regression of intradermally implanted, established tumors. (A) IL-12 cDNA gene construct engineered in the pWRG 3169 expression plasmid. CMV, cytomegalovirus i/e promoter; sd/sa, the SV40 splicing donor/splicing acceptor site; bGH PA, bovine growth hormone polyadenylation signal sequence. (B) Detection of transgenic IL-12 protein in gene gun-treated skin tissues at 24 h after IL-12 cDNA delivery. Plasmid DNA was precipitated onto 2 μ m gold particles. Mice were shaved in the abdominal area, and the epidermis was transfected with a 300 psi helium gas pulse by using the helium-pulse Accell device (gene gun). Immunoperoxidase assay demonstrates the presence of IL-12 protein (arrows) in the epidermal cell layers of the test mice. (C) Antitumor effect in MethA sarcoma model. Mouse skin overlying and surrounding the target tumor was transfected *in vivo* with IL-12 or Luc cDNA expression vectors on days 7 and 10 after i.d. implantation of 1×10^6 MethA cells. At each treatment, mice received four transfections (5 μ g plasmid DNA/treatment). One transfection was directly over the tumor site, and three additional treatments were evenly spaced around the circumference of the tumor in a triangle pattern. Photographs of test mice were taken on days 7, 17, and 29 after tumor cell implantation. Whereas all mice treated with the control (Luc) plasmid DNA developed large tumors, three of eight mice treated with IL-12 gene exhibited complete tumor regression as shown here; the other five mice had reduced tumor growth.

graded numbers of viable effector cells and ^{51}Cr -labeled Renca cells (10^4) were placed into the round-bottomed wells of 96-well plates. After incubating for 4 hr at 37°C , radioactivity in supernatants was determined.

RESULTS AND DISCUSSION

Transgenic Expression of IL-12 *in Vitro* and *in Vivo*. We constructed a plasmid (pWRG3169) containing coding sequences for the p35 and p40 subunits of mIL-12, linked tandemly in the same direction and each driven by its own CMV i/e promoter/enhancer, a SV40 sd/sa intron sequence, and a bovine growth hormone polyadenylation sequence (Fig. 1A). This version of a mIL-12 vector was found to be 3- to 8-fold more efficient in expressing IL-12 protein in B16 tumor cells transfected *in vitro* or in murine skin transfected *in vivo* than the same cDNA clones constructed in a single operon with an internal ribosome entry site linkage. This vector was also more efficient than using gold beads coated with a mixture of two different expression plasmids, one for each IL-12 subunit (data not shown). *In vitro* and *in vivo* expression of IL-12 was performed with this tandem IL-12 gene construct and compared with expression by a control vector containing a Luc cDNA expression plasmid. The level of transgenic IL-12 was determined by a cell proliferation bioassay. Upon *in vitro* gene gun-mediated delivery of $1.25\ \mu\text{g}$ pWRG3169 DNA into 1×10^6 B16 (murine melanoma) cells, $49.8 \pm 10.2\ \text{ng}$ of functionally active IL-12 were detected at 24 h posttransfection. At 24 h after *in vivo* gene transfer into skin tissue, $266.0 \pm 27.8\ \text{pg}$ of mIL-12 were detected per $0.172 \pm 0.026\ \text{g}$ of fresh weight tissue within a standard $1.5 \times 1.5\ \text{cm}^2$ full thickness skin biopsy that contained four gene gun-treated sites. Because of the limited sensitivity of the current IL-12 bioassay for serum and skin tissue extracts ($\geq 100\ \text{pg/ml}$), we were unable to detect the low levels of IL-12 that might have been released into serum of test mice. We have previously shown (14) that very low levels of other cytokines, such as interferon- γ , interleukin 6, or granulocyte/macrophage colony-stimulating factor, can be detected in serum of mice undergoing gene therapy on the skin. It is important to note that the amount of IL-12 detected in the *in vivo* skin transfection sites was 1/400 to 1/40,000 of the dosage ($0.1\text{--}10\ \mu\text{g}$) of the systemically injected rIL-12 protein which resulted in both antitumor effects and toxicity in mice (5–7).

Skin tissue overlying a 7-day i.d. Renca tumor was treated with IL-12 expression plasmid by gene gun delivery and biopsied 24 h later. Histologic examination revealed that the gold particles primarily penetrated to the epidermal cell layers of the mouse skin tissue but not into the underlying tumor cells. Accordingly, immunohistochemical staining of the skin tissue 24 h following gene gun delivery with pWRG3169 revealed that transgenic IL-12 was expressed only in the epidermal cell layers (Fig. 1B).

Tumor Regression and Suppression of Tumor Growth Following IL-12 Gene Therapy. It is known that certain murine immunogenic tumors can induce a T cell-mediated immune response that is best detected on days 7–9 of tumor growth in defined tumor models (22–24). Therefore we started the IL-12 cDNA treatments at 7 days postimplantation of tumor cells with the hope of enhancing the already activated endogenous antitumor immune response. Using this experimental strategy, the *in vivo* delivery of the chimeric IL-12 genes into skin tissues overlying established 7-day tumors resulted in complete tumor regression or suppression of tumor growth in four tumor models (Figs. 1C and 2). In mice bearing Renca, L5178Y, MethA, or SA-1 tumors, complete tumor regression was achieved in 87.5% (7/8), 87.5% (7/8), 57% (4/7), and 37.5% (3/8) of the tested mice, respectively (Fig. 2). Nearly identical results were achieved with Renca tumors after a single IL-12 cDNA treatment on day 7 (data not shown). In contrast,

tumors grew progressively in most of the untreated mice (data not shown) or mice treated with Luc gene (Fig. 2). Furthermore, in mice bearing P815 mastocytoma or B16 melanoma, a significant suppression of tumor growth was achieved (Fig. 2). For example, on day 13 post-P815 tumor cell implantation, the mean tumor diameter in mice treated with IL-12 gene was $8.89 \pm 0.27\ \text{mm}$ versus $12.28 \pm 0.46\ \text{mm}$ in mice treated with Luc control gene in the same expression plasmid ($P < 0.001$). Likewise, on day 15 post-B16 tumor cell implantation, tumor diameter in mice treated with IL-12 gene was $6.30 \pm 0.45\ \text{mm}$ versus $11.8 \pm 0.31\ \text{mm}$ in mice treated with Luc gene ($P < 0.001$). However, the observed suppression of tumor growth was transient and all mice eventually died from progressing tumors. Whether or not a modification in gene transfer schedules could improve the result of therapy of these two weakly immunogenic tumors is unclear and warrants further evaluations, especially since most human tumors are believed to be weakly or not immunogenic.

It is important to note that for all tested mouse tumor models, the tumors were already well established at the beginning of the therapy, and had reached 5–8 mm in diameter. To our knowledge, this is the first evidence that an IL-12 gene therapy protocol can cause a complete regression of

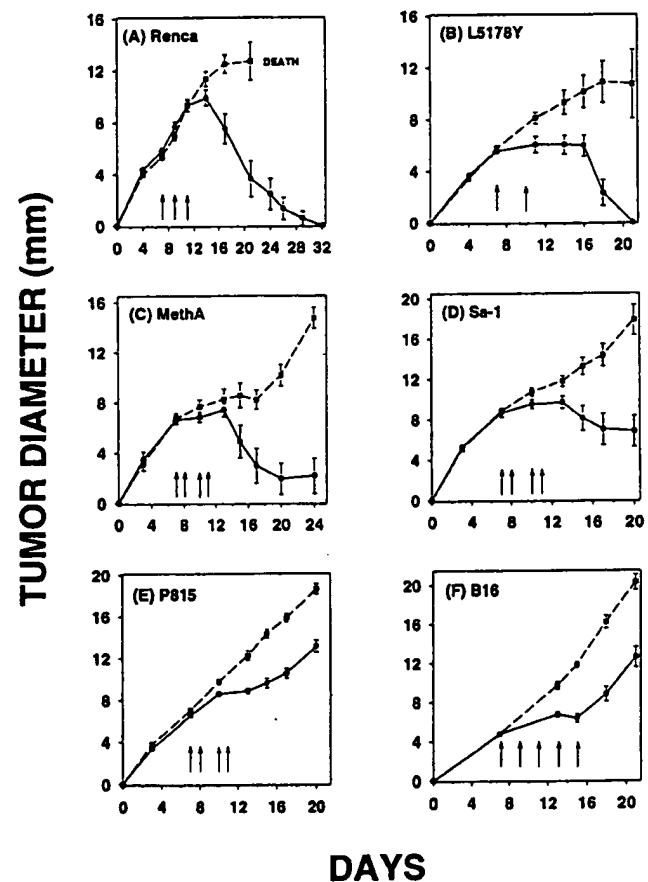


FIG. 2. Kinetics of regression of established murine tumors following *in vivo* IL-12 gene therapy. The gene therapy procedure was started at 7 days after i.d. injection of 1×10^6 (or 1×10^5 , in the case of B16 tumor) indicated tumor cells. At each treatment, mice received four transfections with IL-12 DNA (circles) or with control DNA, pCMVLuc (squares). The arrows on each graph indicate the days following tumor injection on which gene transfer treatments were carried out. Mean tumor diameters \pm SEM are shown for 7–8 mice per group except for B16 tumor model (12 mice per group). The IL-12 gene therapy experiments were repeated five times with the Renca tumor system, two times with MethA and P815 tumors, and once with L5178Y and B16 tumor models, and similar results were obtained.

established, relatively large tumors. Previous studies have shown that IL-12 gene therapy using retroviral vectors resulted in prevention of tumor development (9), or regression of small, 3-day-old MCA207 sarcomas in 33% of treated mice (10). It is also noteworthy that only 1–4 days of therapy (using four gene gun treatments per tumor site on each day of therapy) resulted in tumor regression or growth suppression in virtually all of our experiments. In previous studies using recombinant protein therapy, tumor regression required daily injections of IL-12 at doses from 0.1 to 10 μg for 1 week (6), or 5 days a week for 3–4 weeks (5).

It is also important to note that in our IL-12 gene therapy protocol, the normal skin tissue overlying an established tumor is intentionally transfected topically to incite the existing antitumor immune response. The results presented in Figs. 1 and 2, in conjunction with our previous findings using other cytokine genes (14), indicate that transgenic IL-12 production by normal epidermal cells in the vicinity of the tumor is responsible for the antitumor effect of IL-12 gene therapy. Therefore, the *in vivo*, particle-mediated IL-12 gene transfer protocol is drastically different from other currently employed procedures of cancer gene therapy, where a therapeutic vector is either introduced into the tumor or other cells *in vitro*, or injected directly into the tumor mass *in vivo*.

Involvement of CD8⁺ T Cells in Tumor Regression. To determine the role of CD4⁺ and CD8⁺ T cells in the observed tumor regression, we injected Renca tumor-bearing mice with anti-CD4 or anti-CD8 mAb on the next day after the beginning of IL-12 gene therapy and then 4 days later. This protocol was based on our previous findings showing that the same mAbs caused depletion of more than 90% of relevant T cell subsets in mice for 4–5 days following a single injection (25, 26). Fig. 3 provides direct evidence that the IL-12 gene therapy-induced tumor regression required CD8⁺ cells, in that *in vivo* depletion of CD8⁺ T cells, but not the depletion of CD4⁺ T cells, abrogated the effect of IL-12 gene therapy. These data are in agreement with the findings of Brunda *et al.* (5) that tumor regression caused by rIL-12 is mediated by CD8⁺ T cells, and

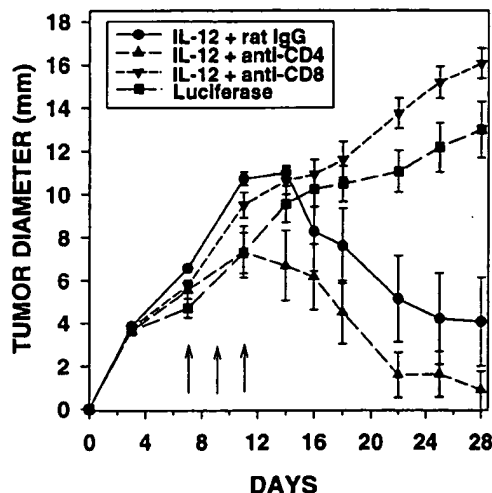


FIG. 3. Tumor regression caused by IL-12 gene requires CD8⁺ T cells. BALB/c mice were injected i.d. with 1×10^6 Renca cells. Skin was transfected with IL-12 or Luc cDNA expression vectors on days 7, 9, and 11 posttumor implantation (arrows). Anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 2.43), both obtained from the Trudeau Institute (Saranac Lake, NY), were administered intraperitoneally on days 8 (300 $\mu\text{g}/\text{mouse}$) and 12 (150 $\mu\text{g}/\text{mouse}$) after tumor implantation. Control groups included mice that were treated with the IL-12 gene and received rat IgG (Sigma) at the same doses and schedule as the anti CD8- and CD4 mAb, or mice treated with the Luc gene instead of the IL-12 gene. Mean tumor diameters \pm SEM are shown for eight mice per group.

not by CD4⁺ T cells. In fact, depletion of CD4⁺ T cells with anti-CD4 mAb appeared to result in slightly accelerated tumor regression (Fig. 3), implying that CD4⁺ T cells may have suppressed the anti-tumor effect of IL-12 in this tumor model. Indeed, it has been shown that established tumors induce Th2-like CD4⁺ T suppressor cells, which can inhibit CD8⁺ T cell-mediated immune responses (24, 27, 28). The beneficial effect of anti-CD4 mAb treatment for tumor immunotherapy with rIL-2 protein (25) or IL-12 gene (29) has been previously reported. Supporting data show that IL-12 protein can activate tumor-specific CD8⁺ T cells *in vitro* (30) and mediate an anti-suppressive effect on Th2 CD4⁺ T cells *in vivo* (31, 32).

Anti-Metastatic Effect of Local IL-12 Gene Therapy. The results showing that tumor regression caused by local IL-12 gene therapy requires CD8⁺ T cells suggest that local IL-12 gene delivery might result in a systemic antitumor effect. To test this hypothesis, we used the P815 tumor cells that metastasize into the visceral organs several days after the i.d. implantation, thereby causing the death of the mice even when the primary tumor has been surgically removed (20). In this system, an excision of the primary P815 tumor on day 12 (data not shown) or day 15 (Fig. 4) posttumor implantation was followed by death of all treated mice by day 37 or 29, respectively. However, when the skin overlying the i.d. P815 tumors were transfected with IL-12 cDNA on days 12 and 14 posttumor cell implantation, followed by tumor excision on day 15, and three additional IL-12 cDNA skin transfections at the abdominal sites adjacent to the excised primary tumor, a significant prolongation of the survival was observed (Fig. 4). These results suggest that local delivery of IL-12 gene into the skin tissue overlying and surrounding the primary tumor can

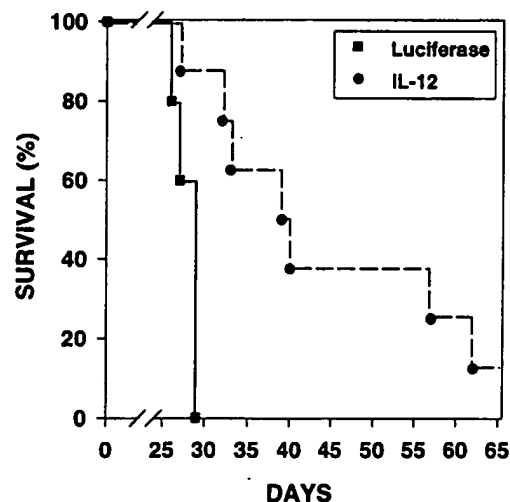


FIG. 4. Anti-metastatic effect of IL-12 gene therapy on P815 tumors. DBA/2 mice were injected i.d. with 1×10^6 P815 cells. Skin tissues overlying and surrounding the target tumor were treated with IL-12 cDNA delivered by gene gun (8 mice/group) or Luc cDNA (5 mice/group) on days 12 and 14 after tumor cell implantation. Surgical excision of the tumor was performed as described (20) on day 15, when tumor size reached about 13 mm in diameter. Additional transfections of skin on both sides of the abdomen were performed on days 16, 18, and 20 after implantation of tumor cells. All mice treated with the control Luc cDNA died in 28.0 ± 0.6 days after tumor cell implantation. Death was caused by spontaneous metastases of tumor cells into the internal organs, primarily the liver, as was evidenced by macroscopic examination (data not shown). IL-12 gene therapy effectively prolonged the survival of mice (survival time 41.4 ± 4.9 days, $P < 0.05$), and one of eight mice was "cured." This experiment was repeated without additional transfections posttumor excision, and we observed that all of the Luc cDNA treated mice ($n = 11$) died in 43.9 ± 7.1 days, whereas 5 of 12 (41.6%) IL-12 gene therapy-treated mice survived for at least 180 days and thus were considered cured.

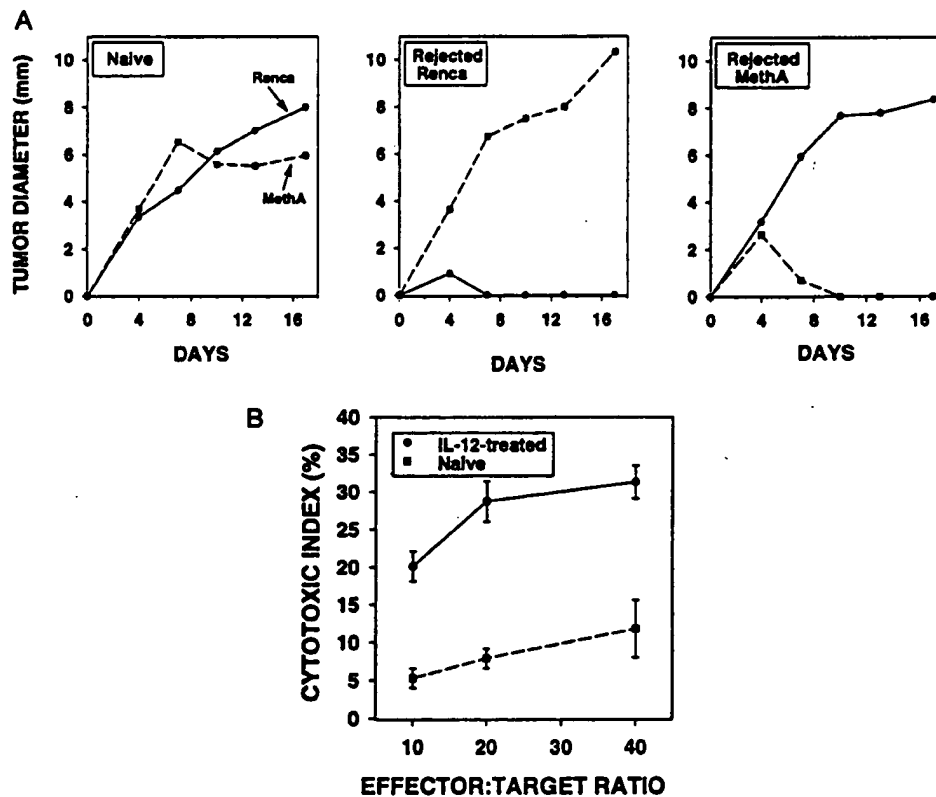


FIG. 5. IL-12 gene therapy in tumor-bearing mice results in development of tumor-specific immunological memory. (A) Rejection of secondary tumor challenge following IL-12 gene therapy. BALB/c mice that rejected Renca or MethA tumors following IL-12 gene therapy were injected one month later with 1×10^6 of both Renca cells (circles) and MethA cells (squares) on the right and the left side of abdomen, respectively. As a control, the tumor cells were injected into age-matched naive BALB/c mice. Data are presented as the means of five to eight mice per group. The experiment was repeated using mice that rejected L5178Y tumors and were secondarily challenged with L5178Y or P815 tumor cells, and similar results were obtained. (B) Induction of CTL activity in mice that rejected tumors following IL-12 gene therapy. Tumor-specific CTL were generated *in vitro* as described. Mean \pm SEM of four mice per group. Spleen cells from IL-12 gene-treated mice generated 3- to 4-fold higher levels of CTL activity than spleen cells from naive mice ($P < 0.005$).

augment systemic antitumor immune response even against a weakly immunogenic tumor, and this can lead to eradication of established spontaneous metastases in mice. Therefore, such human metastatic cancers as subcutaneous T-cell lymphoma or melanoma may provide excellent models for future clinical application of the current IL-12 gene therapy approach.

Immunological Memory in Mice Following IL-12 Gene Therapy. It has been recently shown that tumor regression caused by rIL-12 protein therapy results in development of a memory immune response against the tumor (33). We evaluated if the mice that rejected tumors following the *in vivo* IL-12 gene therapy developed tumor-specific immunity. Fig. 5A shows that the mice which rejected Renca tumors and were tumor-free for 1 month resisted a second challenge with Renca cells but developed tumors when challenged with MethA tumor cells. Inversely, the mice that rejected MethA tumors following IL-12 gene therapy resisted the second challenge with MethA cells, but developed tumors when challenged with Renca cells. These results demonstrate that mice which rejected their tumors following skin transfection with IL-12 gene develop tumor-specific immunological memory against a secondary tumor challenge. Furthermore, spleen cells from the mice that rejected Renca tumors, in contrast to spleen cells from naive mice, exhibited CTL activity upon stimulation with Renca cells *in vitro* (Fig. 5B). In a similar study, using mice that rejected L5178Y tumors, we found that the generated CTL were tumor-specific, in that they lysed the L5178Y target tumor cells but not the syngeneic P815 target cells (data not shown).

Our study shows that gene gun-mediated *in vivo* delivery of an IL-12 expression plasmid into skin tissue overlying tumor sites is an effective approach for tumor immunotherapy in various murine tumor models, leading to eradication or suppression of established intradermal tumors and their spontaneous metastases. Remarkably, the local amount of detectable IL-12 at the treatment tissue site is 1/400 to 1/40,000 of the dosage of rIL-12 protein employed for efficacy studies in the same or similar mouse tumor models (5, 6, 33). In view of the apparent dose dependent toxicity of human rIL-12 protein in clinical trials (8), this dosage difference between the recombinant protein delivered systemically and transgenic protein produced via *in vivo* DNA delivery may make the present IL-12 gene therapy approach more favorable for clinical considerations. Given the efficacy, simplicity, and potential cost-effectiveness of the gene gun-mediated IL-12 gene therapy approach, further preclinical development of this approach is warranted in order to consider initiation of human cancer clinical trials.

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1. Kobayashi, M., Fitz, L., Ryan, M., Hewick, R. M., Clark, S. C., Chang, S., Koudon, R., Sherman, F., Perussia, B. & Trinchieri, G. (1989) *J. Exp. Med.* 170, 827-845.

2. Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Young, H. A., Wolf, S. G., Young, D., Clark, S. C. & Trinchieri, G. (1991) *J. Exp. Med.* **173**, 869–879.
3. Gubler, U., Chua, A. O., Schoenhaut, D. S., Dwyer, C. M., McComas, W., Motyka, R., Mabavi, N., Wolitzky, A. G., Quinn, P. M., Familletti, P. C. & Gately, M. K. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4143–4147.
4. Wolf, S. F., Temple, P. A., Kobayashi, M., Young, E., Dicig, M., Lowe, L., Dzialo, R., Fitz, L., Ferenz, C., Hewick, R. M., Kelleher, K., Herrmann, S. H., Clark, S. C., Azzoni, L., Chan, S. H., Trinchieri, G. & Perussia, B. (1991) *J. Immunol.* **146**, 3074–3081.
5. Brunda, M., Luistro, L., Warrior, R., Wright, R., Hubbard, B., Murphy, M., Wolf, S. & Gately, M. (1993) *J. Exp. Med.* **178**, 1223–1230.
6. Nastala, C., Edington, H., McKinney, T., Tahara, H., Nalesnik, M., Brunda, M., Gately, M., Wolf, S., Schreiber, R., Storkus, W. & Lotze, M. (1994) *J. Immunol.* **153**, 1697–1706.
7. Orange, J., Salazar-Mather, T., Opal, S., Spencer, R., Miller, A., McEwin, B. & Biron, C. (1995) *J. Exp. Med.* **181**, 901–914.
8. Marshall, E. (1995) *Science* **268**, 1555.
9. Tahara, H., Zeh, H., Storkus, J., Pappo, I., Watkins, S., Gubler, U., Wolf, S., Robbins, P. & Lotz, M. (1994) *Cancer Res.* **54**, 182–189.
10. Tahara, H., Zitvogel, L., Storkus, W., Zeh, H., III, McKinney, T., Schreiber, R., Gubler, U., Robbins, P. & Lotze, M. (1995) *J. Immunol.* **154**, 6466–6474.
11. Yang, N. S. & Sun, W. (1995) *Nat. Med.* **1**, 481–483.
12. Cheng, L., Ziegelhoffer, P. & Yang, N. S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4455–4459.
13. Yang, N. S., DeLuna, C. & Cheng, L. (1994) in *Gene Therapeutics: Methods and Applications of Direct Gene Transfer*, ed. Wolff, J. A. (Birkhauser, Boston), pp. 193–209.
14. Sun, W., Burkholder, J., Sun, J., Culp, J., Turner, J., Lu, X., Pugh, T., Ersler, W. & Yang, N. S. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2889–2893.
15. Desal, B., Quinn, P., Wolitzky, A., Mongini, P., Chizzonite, R. & Gately, M. (1992) *J. Immunol.* **148**, 3125–3132.
16. Zeh, H., III, Hurd, S., Storkus, W. & Lotze, M. (1993) *J. Immunother.* **14**, 155–161.
17. National Institutes of Health (1985) *Guide for Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD), NIH Publ. 86–23.
18. Rajagopalan, L., Burkholder, J., Turner, J., Culp, J., Yang, N. S. & Malter, J. (1995) *Blood* **86**, 2551–2558.
19. Schoenhaut, D., Chua, A., Wolitzky, A., Quinn, P., Dwyer, C., McComas, W., Familletti, P., Gately, M. & Gubler, U. (1992) *J. Immunol.* **148**, 3433–3440.
20. Dye, E. S. (1986) *J. Immunol.* **136**, 1510–1515.
21. Rakhmilevich, A. L., North, R. J. & Dye, E. S. (1993) *Int. J. Cancer* **55**, 338–343.
22. North, R. J. (1984) *Cancer Immunol. Immunother.* **18**, 69–74.
23. North, R. J. & Dye, E. S. (1985) *Immunology* **54**, 47–56.
24. North, R. J. & Bursucker, I. (1984) *J. Exp. Med.* **159**, 1295–1311.
25. Rakhmilevich, A. L. & North, R. J. (1994) *Cancer Immunol. Immunother.* **38**, 107–112.
26. Rakhmilevich, A. L. (1994) *Immunology* **83**, 524–531.
27. Mills, C. & North, R. J. (1985) *Transplantation* **39**, 202–208.
28. Bear, H. D. (1986) *Cancer Res.* **46**, 1805–1812.
29. Martinotti, A., Stoppacciaro, A., Vagiani, M., Melani, C., Spreafico, F., Wysocka, M., Parmiani, G., Trinchieri, G. & Colombo, M. (1995) *Eur. J. Immunol.* **25**, 137–146.
30. Andrews, J. V. R., Schoof, D., Bertagnolli, M., Peoples, G., Goedegebuure, P. & Eberlein, T. (1993) *J. Immunother.* **14**, 1–10.
31. Schmitt, D., Owen-Schaub, L. & Ullrich, S. (1995) *J. Immunol.* **154**, 5114–5120.
32. Nabors, G., Afonso, L., Farrell, J. & Scott, P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 3142–3146.
33. Mu, J., Zou, J.-P., Yamamoto, N., Tsutsui, T., Tai, X.-G., Kobayashi, M., Herrmann, S., Fujiwara, H. & Hamaoka, T. (1995) *Cancer Res.* **55**, 4404–4408.



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In vivo gene gun-mediated transduction into rat heart with Epstein-Barr virus-based episomal vectors

Kazuhiko Nishizaki, Osam Mazda, Yoshiko Dohi, Tetsuji Kawata, Kazumi Mizuguchi,
Soichiro Kitamura and Shigeki Taniguchi
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In Vivo Gene Gun–Mediated Transduction Into Rat Heart With Epstein-Barr Virus-Based Episomal Vectors

Kazuhiko Nishizaki, MD, Osam Mazda, MD, PhD, Yoshiko Dohi, PhD, Tetsuji Kawata, MD, Kazumi Mizuguchi, MD, Soichiro Kitamura, MD, and Shigeki Taniguchi, MD

Departments of Surgery III and Public Health, Nara Medical University, Nara, Department of Microbiology, Kyoto Prefectural University of Medicine, Kyoto, and Division of Cardiovascular Surgery, National Cardiovascular Center, Osaka, Japan

Background. Gene guns have been used to transfer genes into various organs, but there has been no report of successful gene gun–mediated gene transfer into the heart. In this study, we assessed the possibility of gene therapy using a gene gun and an episomal plasmid vector.

Methods. Gene transfer was performed using two sizes of gold particles and two plasmids (an episomal vector and a conventional plasmid vector). From the first to eighth week after the bombardment, rats were sacrificed. The excised hearts were subjected to X-gal staining and histologic examination. To ensure that plasmid was not distributed to organs other than the heart, the presence of

the β -gal sequence was examined by polymerase chain reaction analyses.

Results. Gene expression persisted for 6 weeks. The episomal vector apparently contributed to long-lasting expression. Infiltration of monocytes or leukocytes was very faint. The β -gal DNA was detected in bombarded hearts but not other organs.

Conclusions. Gene gun–mediated transfer of the episomal vector into beating heart may provide a simple, efficient, and useful strategy for gene therapy.

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In vivo gene transfer into the beating heart is an attractive strategy for gene therapy for cardiovascular diseases. Many techniques for gene transfer have been reported, including direct injection of naked plasmid DNA and infusion of various viral or nonviral vectors. In this study, we tested the possibility of direct in vivo transfer of plasmid DNA into heart by a gene gun.

In a gene gun system, micron-sized gold particles are coated with plasmid DNA and then accelerated at high velocity toward target cells or tissues. Cells penetrated by these particles have a high likelihood of being transfected by the DNA thus introduced. Gold and tungsten particles are commonly used as the carrier of plasmid DNA. Because of the high specific gravity and small diameter, these particles easily penetrate into cells. Also, they are not cytotoxic. The gene gun was first devised to transfect plant cells, the walls of which act as a physical barrier to conventional transfection techniques [1]. More recently, it was demonstrated that gene transfer into various mammalian tissues could also be successfully achieved by the gene gun. These tissues include liver, skin, skeletal muscle [2], and pancreas [3]. To our knowledge, however, heart has not yet been targeted.

We employed an Epstein-Barr virus (EBV)-based epi-

somal vector to obtain long-lasting transgene expression in vivo. The EBV-based episomal vector is a plasmid vector carrying oriP and the EBV nuclear antigen 1 (EBNA1) gene from EBV. The EBNA1 gene facilitates the maintenance of the episomes through binding to oriP. After being transfected into human cells, the plasmid persists extrachromosomally at low copy numbers owing to replication and nuclear retention of plasmid DNA [4].

We show here that gene gun–mediated transfer of the EBV-based episomal vector into rat heart results in long-lasting expression of a marker gene.

Material and Methods

Animals and Surgery

Male Wistar rats were used for this study. All rats were between 10 and 12 weeks old. They received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Institutes of Health publication 86 to 23, revised 1985). After being anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal injection), rats were intubated and ventilated with room air. An anterolateral thoracotomy was performed at the location of most pronounced cardiac pulsation and beating hearts were exposed. The pericardium was stripped, and the right chest and abdomen were pressed to push the heart out of the thoracic cavity. After bombardment in

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Address reprint requests to Dr Nishizaki, Department of Surgery III, Nara Medical University, Kashihara, Nara 634–8522, Japan; e-mail: nszk@naramed-u.ac.jp.

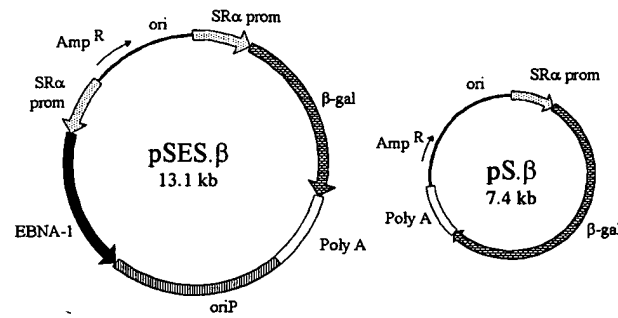


Fig 1. Plasmids used in this study. Maps of pSES.β (left) and pS.β (right) are shown. (Prom = promoter, polyA = SV40 polyA additional signal.)

the anterior wall of left ventricular near the apex, a chest tube was placed to drain air and fluids, and the wound was closed in two layers.

Plasmids

The plasmid vectors, pSES.β and pS.β, were previously described [5]. Briefly, pSES.β (Fig 1, left) is composed of the *Escherichia coli* β-gal gene located between SRα promoter and the SV40 polyA additional signal, EBV oriP, EBV EBNA1 gene under control of the SRα promoter, the ampicillin resistance gene, and the replication origin for *E. coli*. The other plasmid, pS.β (Fig 1, right), was constructed from pSES.β by deleting EBNA1 and oriP.

Bombardment

We used a gene delivery system (model TF-1) designed by Nihon Medical & Chemical Instruments Co, Ltd, (Osaka, Japan). With this device, high-pressure helium provides the motive force for gold particles. The size of gold particles influences the transduction efficiency as well as the degree of tissue damage. Although the larger particles reach the deeper layer, cell damage could be more serious. Therefore, two sizes of gold particles were examined (1.0 Au and 1.5 Au: 1.0 μm and 1.5 μm in diameter, respectively). Supercoiled plasmids were precipitated gold particles at a ratio of 20 μg DNA:5 mg gold particles. The DNA-coated particles were suspended in 500 μL ethanol, and 20 μL of the suspension was distributed on a 25-mm titanium plate (0.5-mm thickness). Each rat received two consecutive bombardments in the anterior wall of left ventricular near the apex. The size of bombarded area was about 0.5 to 1.0 cm². The helium pressure was set at 1.05 MPa, and the distance between the surface of the heart and the titanium plate was 2 cm. As a control group, 10 rats (1.0 Au, n = 5; 1.5 Au, n = 5) were bombarded with uncoated gold particles.

Histologic Analysis

For histologic examination, animals were sacrificed 1, 2, 3, 4, 6, or 8 weeks after bombardment. An 18-gauge catheter was inserted into the abdominal aorta and a median sternotomy was performed. After the pulmonary artery and inferior vena cava were cut, the heart was perfused with 50 mL iced PBS followed by 20 mL 4%

paraformaldehyde (PFA) in a retrograde manner using this catheter. The hearts were excised, sliced (500 μm thick) by a vibratome slicer (DOSAKA, Kyoto, Japan), and refixed in 4% PFA for 1 hour. After washing three times in PBS at room temperature, all sections were incubated overnight at 37°C in X-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactosidase [X-gal], 3 mmol/L K₄[Fe(CN)₆], 3 mmol/L K₃[Fe(CN)₆], 1 mmol/L MgCl₂, and 0.1% Triton X-100 in PBS). After being washed in PBS, the samples were refixed overnight with 4% PFA at 4°C, dehydrated in ethanol, and embedded in paraffin. We judged whether the bombarded portion of the heart stained blue by microscopic observations.

Polymerase Chain Reaction

For polymerase chain reaction (PCR) testing, DNA was isolated from the lung, brain, liver, spleen, kidney and heart of animals 7 days after bombardment. Synthetic oligodeoxynucleotide primers were prepared corresponding to the β-gal gene sequence (sense primer: 5'-GCC GAC CGC ACG CCG CAT CCA GC-3', anti-sense primer: 5'-CGC CGC GCC ACT GGT GTG GGC C-3') [6]. The reaction mixture consisted of 1 mg DNA, 1.5 mmol/L MgCl₂, 2 mmol/L dNTP, 0.5 μmol/L of each oligonucleotide primer, and 2.5 U of Taq DNA polymerase (Amersham Pharmacia Biotech Ltd, Uppsala, Sweden). As positive controls, 5 ng pS.β and pSES.β were also tested. The reaction was performed by Gene Amp 2400 (Perkin Elmer, Norwalk, CT). The amplification profile consisted of 25 cycles of denaturing at 98°C for 15 seconds, annealing at 65°C for 2 seconds, and extension at 74°C for 30 seconds. PCR products were analyzed by polyacrylamide gel electrophoresis.

Results

Bombardment Did Not Affect Rat Survival

We used 133 rats in this study. All rats survived surgery and bombardment procedures, with the exception of 3 rats in early experiments that died of respiratory failure within a few hours after surgery. The remaining rats were maintained on a normal diet until subsequent steps in experiments.

Prolonged Expression of β-Gal in Heart Transduced With EBV-Plasmid by Gene Gun

Two sizes of gold particles and two plasmids (pSES.β and pS.β) were employed (Fig 1). One hundred and twenty rats were divided into four groups, with each group of rats receiving a different combination of plasmid/gold particle (pS.β/1.5 Au, n = 30; pSES.β/1.5 Au, n = 30; pS.β/1.0 Au, n = 30; and pSES.β/1.0 Au, n = 30). Five rats of each group were sacrificed 1, 2, 3, 4, 6, and 8 weeks after bombardment, and their hearts were subjected to X-gal staining and histologic examination.

Seven days after the bombardment, cardiomyocytes in the bombarded portion in all hearts stained blue (Fig 2). The staining layer in bombarded heart was within 1 mm

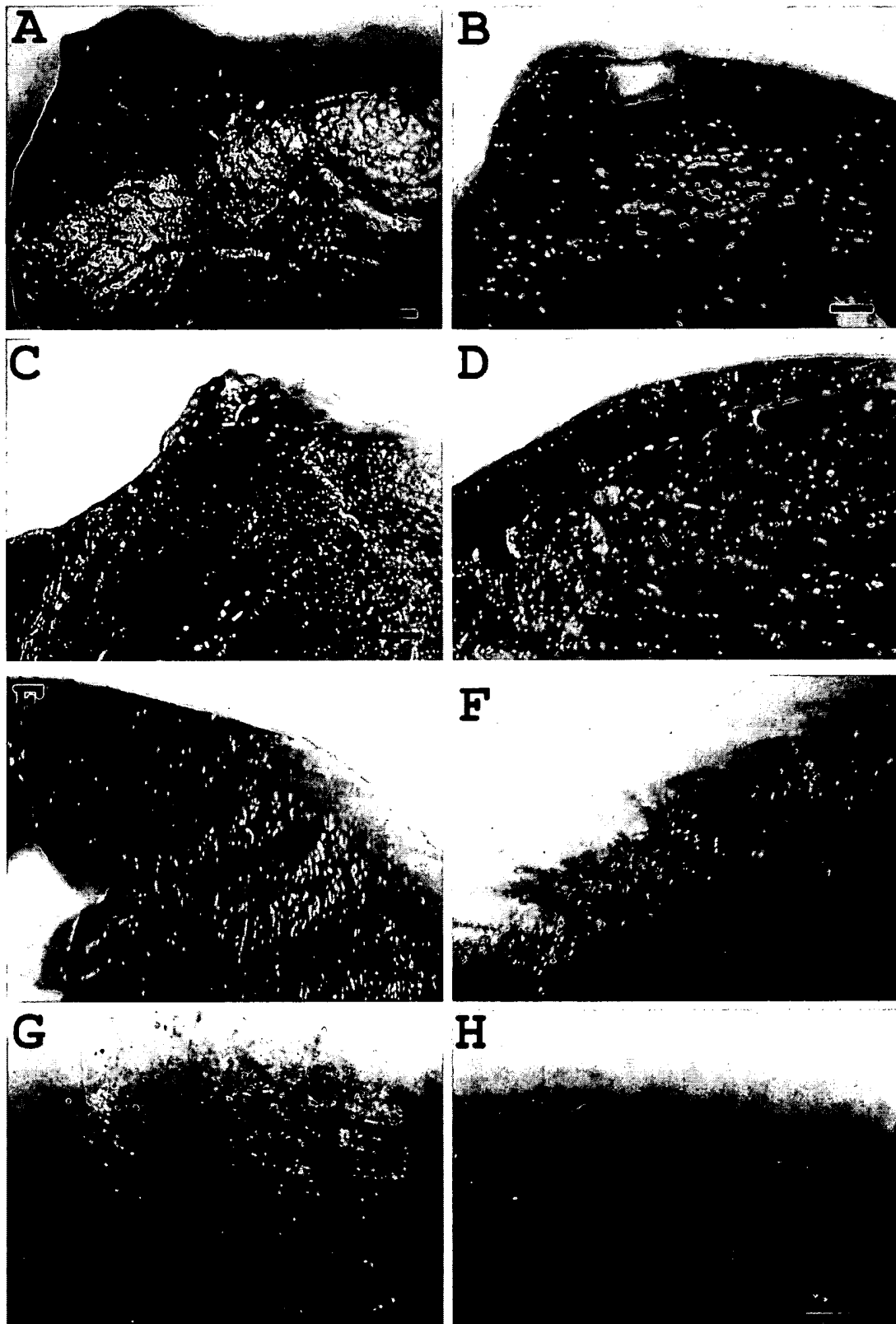


Fig 2. X-gal staining of the bombarded heart. Rat hearts were bombarded with 1.0 Au (A, B, E, and F) or 1.5 Au (C, D, G, and H) gold particles coated with pSES.β (A, C, E, and G) or pS.β (B, D, F, and H). Seven days (A–D) or 4 weeks (E–H) later, rats were sacrificed and the hearts were excised. The hearts were cut into pieces and subjected to X-gal staining as described in Material and Methods. The bar indicates 500 μ m.

Table 1. Period of Gene Expression

Plasmid	Gold Particle	1 Week	2 Weeks	3 Weeks	4 Weeks	6 Weeks	8 Weeks
pSES.β	1.5 Au	5/5 ^a	5/5	5/5	5/5	0/5	0/5
pS.β	1.5 Au	5/5	5/5	5/5	1/5	0/5	0/5
pSES.β	1.0 Au	5/5	5/5	5/5	5/5	5/5	1/5
pS.β	1.0 Au	5/5	5/5	5/5	5/5	1/5	0/5

^a Number of X-gal positive hearts/number of tested hearts.

depth from the surface. Gene expression persisted for 3 weeks (pS.β/1.5 Au), 4 weeks (pSES.β/1.5 Au and pS.β/1.0 Au), or 6 weeks (pSES.β/1.0 Au; Table 1). The hearts of control rats (1.5 Au, n = 5; and 1.0 Au, n = 5) did not stain at all (data not shown). Transfection with an EBV-based episomal vector resulted in more sustained expression of the marker gene than that with a conventional, non-EBV plasmid vector, provided that gold particles of the same size were employed. On the other hand, when the same plasmid was transfected, 1.0 Au was more effective than 1.5 Au, in terms of longevity of gene expression. The combination of 1.0 Au and pSES.β yielded the most prolonged expression in this study. EBV episomal vector apparently contributed to long-lasting expression of β-gal expression.

On histologic examination, many cardiomyocytes in the surface layer stained blue, while few cells stained in deep layers (Fig 2, Fig 3). Probably, DNA-coated gold particles could not reach the deep layers. Some cardiomyocytes carrying DNA-coated gold particles were observed (Fig 3C and D).

Vector DNA Detected in Bombarded Heart but Not in Other Organs

To ensure that plasmid was not distributed to organs other than the heart under the experimental protocol, we

performed PCR and examined the presence of the β-gal sequence. DNA was prepared from the lung, brain, liver, spleen, kidney, and heart of rats in each group sacrificed 7 days after bombardment. The β-gal sequence was detected in bombarded heart but not in other organs or control heart (Fig 4). These findings indicated that the plasmid DNA was present exclusively in the heart.

Comment

In the present study, we showed that plasmid DNA can be introduced into cardiomyocytes using a gene gun and that transfer of the EBV-based episomal vector results in long-lasting gene expression in vivo. To our knowledge, this is the first study showing that in vivo gene transfer into heart can be achieved by a gene gun.

Various gene transfer vectors and delivery methods have been devised to transfer genes into the heart, including direct injection of naked DNA into heart muscle [7], infusion of HVJ-liposomes [8] or cationic liposomes [9] into coronary artery, and injection of HVJ-liposomes into pericardium [10]. Particle-mediated gene transfer has several advantages over other methods. First, the gene gun is a nonviral vector. Side effects associated with viral vectors (eg, generation of aberrantly recombinant, replication-competent viruses) can be avoided. Second, this technique may allow expression of foreign genes in a wide variety of cell types, including terminally differentiated nondividing cells. Third, it can transfer as large a quantity of DNA as can be carried by

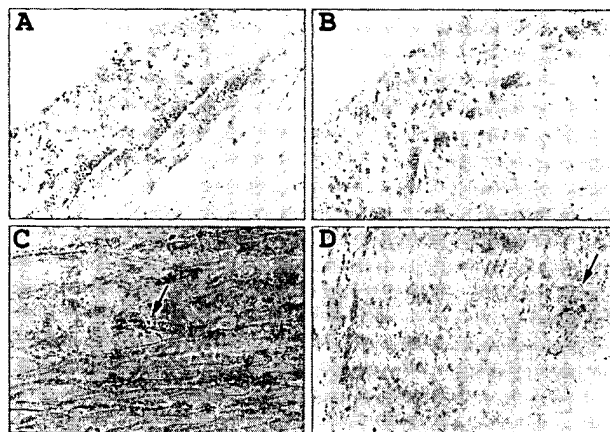


Fig 3. Microscopic observation of bombarded heart stained with X-gal. Rats hearts were bombarded with 1.0 Au (A and B) or 1.5 Au (C and D) gold particles coated with pSES.β (A and C) or pS.β (B and D), and 3 weeks later, rats were sacrificed and the hearts were excised. The heart sections were subjected to X-gal staining and Kernechtrot staining so that nucleus stained pink. The arrow indicates gold particle. Magnification × 400, A–C; × 200, D.

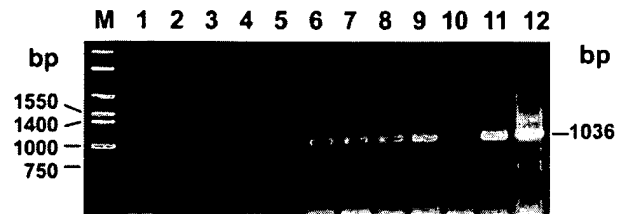


Fig 4. Polymerase chain reaction (PCR) analyses of DNA from various organs. DNA was prepared from the lung (lane 1), brain (lane 2), liver (lane 3), spleen (lane 4), or kidney (lane 5) of a rat whose heart was bombarded with pSES.β/1.0 Au. DNA was also prepared from hearts bombarded with pS.β/1.5 Au (lane 6), pSES.β/1.5 Au (lane 7), pS.β/1.0 Au (lane 8), pSES.β/1.0 Au (lane 9), or 1.0 Au alone (lane 10). As positive controls, 5 ng pS.β (lane 11) and pSES.β (lane 12) were also tested. PCR was performed using β-gal specific primers as described in Material and Methods. (M = molecular weight marker.)

gold particles. Using submicrogram quantities of DNA per bombardment, 1,000 to 10,000 copies of DNA can be delivered into each target cell [11]. Fourth, genes can be introduced exclusively into the bombarded region without being redistributed to other organs.

In particle-mediated transfer, micron-sized gold particles are coated with DNA and accelerated at high velocity toward target cells or tissues. The process may not depend on particular biochemical structure or physical feature of the target cell membrane like liposome/DNA complex-mediated gene transfer [11]. It may be possible to employ a gene gun to transfect cells that are relatively resistant to other delivery systems. For example, it is difficult to transfer genes into infarct areas by coronary infusion, whereas our delivery method may permit perioperative gene transfer into such areas.

In a previous study, we transfected rat cardiac graft *ex vivo* with replication-incompetent adenovirus vector by coronary infusion [12]. After transplantation, massive infiltration of leukocytes was observed close to the transgene-positive cells. It was difficult to avoid the immune responses against the viral vector. In the present study, however, infiltration of monocytes or leukocytes was very faint, if any (Fig 3). No cell damage or inflammatory response was demonstrated by a histologic survey. This is another advantage of gene gun-mediated gene transfer.

We consider that the present method may be useful in treating severe ischemic heart disease by transferring genes to promote angiogenesis, such as basic fibroblast growth factor (bFGF) [13], vascular endothelial growth factor (VEGF) [14], and hepatocyte growth factor (HGF) [15] genes. For patients suffering from severe ischemic heart disease resistant to conventional therapy, transmyocardial laser revascularization (TMLR) is useful. TMLR not only increases the supply of oxygenated blood to the myocardium via left ventricular transmural channels but also induces angiogenesis. If gene gun-mediated transfection of angiogenesis factor genes can be combined with TMLR, angiogenesis may be more efficiently promoted by synergistic action of two systems. Moreover, TMLR may enable gene gun to transfect cells in deeper layers. The combination may greatly contribute to the treatment of patients with various heart diseases, especially severe ischemic heart disease. On the other hand, a gene gun may be equipped on a tip of catheter. Such a device may be feasible for the gene therapy against the coronary stenosis, as well as for endocardial delivery.

We previously demonstrated high transfection efficiency with EBV-based episomal vectors into various human lymphoma cell lines [16-18], hepatocellular carcinoma cell lines [19], primary fibroblasts from skin, bone marrow cells [5], and peripheral blood CD34⁺ cells [20]. Recently, we have also reported that high transient expression was observed in rat heart injected with naked EBV-based plasmid DNA [21]. In the present study, we found that transfection with the EBV-based episomal vector results in more prolonged gene expression in rodent cells than that with conventional plasmid vector.

This is compatible with earlier finding by Saeki and coworkers [22] who reported sustained expression of a marker gene in rat liver injected with EBV-based episomal vector by means of the hemagglutinating virus of Japan (HVJ)-liposome [22]. By our hands, the gene gun was more efficacious than naked plasmid injection. We have two reasons for this high efficiency. First, we employed an episomal plasmid. Second, the gene gun-mediated delivery system enables transfer of the plasmid-coated particles into the nucleus, whereas direct injection of plasmid allows penetration of DNA into cytoplasm but not nucleus.

Direct *in vivo* gene transfer into heart is an attractive strategy for gene therapy. Particle-mediated gene transfer technology provides a physical means of DNA delivery, and the EBV-based episomal vector contributes to stronger and more long-lasting expression. Our findings suggest that the combination of the gene gun and EBV-based vector may be useful for gene therapy of cardiovascular diseases.

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References

1. Klein TM, Wolf ED, Wu R, Sanford JC. High-velocity microprojectiles for delivering nucleic acids into living cells [Letter]. *Science* (London) 1987;327:70-3.
2. Yang NS, Burkholder J, Roberts B, Martinell B, McCabe D. *In vivo* and *in vitro* gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci USA* 1990;87:9568-72.
3. Cheng L, Ziegelhoffer PR, Yang NS. *In vivo* promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proc Natl Acad Sci USA* 1993;90:4455-9.
4. Yates JL, Warren N, Sugden B. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* 1985;313:812-5.
5. Satoh E, Osawa M, Tomiyasu K, et al. Efficient gene transduction by Epstein-Barr-virus-based vectors coupled with cationic liposome and HVJ-liposome. *Biochem Biophys Res Commun* 1997;238:795-9.
6. Csete ME, Drazan KE, van Bree M, et al. Adenovirus-mediated gene transfer in the transplant setting. I. Conditions for expression of transferred genes in cold-preserved hepatocytes. *Transplantation* 1994;57:1502-7.
7. Acsadi G, Dickson G, Love DR, et al. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs [see comments]. *Nature* 1991;352:815-8.
8. Sawa Y, Suzuki K, Bai HZ, et al. Efficiency of *in vivo* gene transfection into transplanted rat heart by coronary infusion of HVJ liposome. *Circulation* 1995;92:479-82.
9. Ellison KE, Bishopric NH, Webster KA, et al. Fusogenic liposome-mediated DNA transfer into cardiac myocytes. *J Mol Cell Cardiol* 1996;28:1385-99.
10. Aoki M, Morishita R, Muraishi A, et al. Efficient *in vivo* gene transfer into the heart in the rat myocardial infarction model using the HVJ (Hemagglutinating Virus of Japan)-liposome method. *J Mol Cell Cardiol* 1997;29:949-59.
11. Yang NS, Sun WH. Gene gun and other non-viral approaches for cancer gene therapy. *Nat Med* 1995;1:481-3.
12. Gojo S, Niwaya K, Taniguchi S, Nishizaki K, Kitamura S. Gene transfer into the donor heart during cold preservation for heart transplantation. *Ann Thorac Surg* 1998;65:647-52.

13. Giordano FJ, Ping P, McKirnan MD, et al. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart [see comments]. *Nat Med* 1996;2:534-9.
14. Muhlhauser J, Merrill MJ, Pili R, et al. VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo. *Circ Res* 1995;77:1077-86.
15. Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Enhanced expression of hepatocyte growth factor/c-Met by myocardial ischemia and reperfusion in a rat model [see comments]. *Circulation* 1997;95:2552-8.
16. Mazda O, Teshigawara K, Fujimoto S, et al. A reporter system using a flow cytometer to detect promoter/enhancer activity in lymphoid cell lines. *J Immunol Meth* 1994;169:53-61.
17. Hirai H, Satoh E, Osawa M, et al. Use of EBV-based vector/HVJ-liposome complex vector for targeted gene therapy of EBV-associated neoplasms. *Biochem Biophys Res Commun* 1997;241:112-8.
18. Mazda O, Satoh E, Yasutomi K, Imanishi J. Extremely efficient gene transfection into lympho-hematopoietic cell lines by Epstein-Barr virus-based vectors. *J Immunol Meth* 1997;204:143-51.
19. Harada Y, Iwai M, Tanaka S, et al. High efficient suicide gene expression in hepatocellular carcinoma cells Epstein-Barr virus-based plasmid vectors combined with polyamido-amine dendrimer. *Cancer Gene Ther* 2000;7:27-36.
20. Satoh E, Hirai H, Inaba T, et al. Successful transfer of adenosine deaminase (ADA) gene in vitro into human peripheral blood CD34+ cells by transfecting Epstein-Barr virus (EBV)-based episomal vectors. *FEBS Lett* 1998;441:39-42.
21. Tomiyasu K, Satoh E, Oda Y, et al. Gene transfer in vitro and in vivo with Epstein-Barr virus-based episomal vector results in markedly high transient expression in rodent cells. *Biochem Biophys Res Commun* 1998;253:733-8.
22. Saeki Y, Wataya-Kaneda M, Tanaka K, Kaneda Y. Sustained transgene expression in vitro and in vivo using an Epstein-Barr virus replicon vector system combined with HVJ liposomes. *Gene Ther* 1998;5:1031-7.

New Requirements for Recertification/Maintenance of Certification in 2001

Diplomates of the American Board of Thoracic Surgery who plan to participate in the recertification/maintenance of certification process in 2001 should pay particular attention to this notice because the requirements have changed.

In addition to an active medical license and institutional clinical privileges in thoracic surgery, effective in 2001, a valid certificate is an absolute requirement for entrance into the recertification/maintenance of certification process. If your certificate expired, the only pathway for renewal of a certificate will be to take and pass the Part I (written) and the Part II (oral) certifying examinations.

In 2001, the American Board of Thoracic Surgery will no longer publish the names of individuals who have not recertified. In the past, a designation of "NR" (not recertified) was used in the American Board of Medical Specialties directories if a Diplomate had not recertified. The Diplomate's name will be published upon successful completion of the recertification/maintenance of certification process.

The CME requirements have also changed. The new CME requirements are 70 Category I credits in either cardiothoracic surgery or general surgery earned during the 2 years prior to application. SESATS and SESAPS are the only self-instructional materials allowed for credit. Category II credits are not allowed. The Physicians Recognition Award for recertifying in general surgery is not allowed in fulfillment of the CME requirements. Interested individuals should refer to the 2001 *Booklet of Information* for a complete description of acceptable CME credits.

Diplomates should maintain a documented list of their major cases performed during the year prior to application for recertification. This practice review should consist of 1 year's consecutive major operative experiences. If more than 100 cases occur in 1 year, only 100 should be listed.

Candidates for recertification/maintenance of certification will be required to complete both the general thoracic and the cardiac portions of the SESATS self-assessment examination. It is not necessary for candidates to purchase SESATS individually because it will be sent to candidates after their application has been approved.

Diplomates may recertify the year their certificate expires, or if they wish to do so, they may recertify up to two years before it expires. However, the new certificate will be dated 10 years from the date of expiration of their original certificate or most recent recertification certificate. In other words, recertifying early does not alter the 10-year validation.

Recertification/maintenance of certification is also open to Diplomates with an unlimited certificate and will in no way affect the validity of their original certificate.

The deadline for submission of applications for the recertification/maintenance of certification process is May 1 each year. A brochure outlining the rules and requirements for recertification/maintenance of certification in thoracic surgery is available upon request from the American Board of Thoracic Surgery, One Rotary Center, Suite 803, Evanston, IL 60201; telephone number: (847) 475-1520; fax: (847) 475-6240; e-mail: abts@evanston.msn.com.

In vivo gene gun-mediated transduction into rat heart with Epstein-Barr virus-based episomal vectors

Kazuhiko Nishizaki, Osam Mazda, Yoshiko Dohi, Tetsuji Kawata, Kazumi Mizuguchi,
Soichiro Kitamura and Shigeki Taniguchi
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**THE ANNALS OF
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***In vivo* cytokine gene transfer by gene gun reduces tumor growth in mice**

WENN H. SUN*†, JOSEPH K. BURKHOLDER‡, JIAN SUN‡, JERILYN CULP‡, JOEL TURNER‡, XING G. LU*, THOMAS D. PUGH*, WILLIAM B. ERSHLER*, AND NING-SUN YANG†§§

*Department of Medicine, Institute on Aging, and †Department of Pathology, University of Wisconsin, 1300 University Avenue, Madison, WI 53706; and ‡Department of Cancer Gene Therapy, Agracetus Inc., 8520 University Green, Middleton, WI 53562

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ABSTRACT Implantation of tumor cells modified by *in vivo* cytokine gene transfer has been shown by many investigators to result in potent *in vivo* antitumor activities in mice. Here we describe an approach to tumor immunotherapy utilizing direct transfection of cytokine genes into tumor-bearing animals by particle-mediated gene transfer. *In vivo* transfection of the human interleukin 6 gene into the tumor site reduced methylcholanthrene-induced fibrosarcoma growth, and a combination of murine tumor necrosis factor α and interferon γ genes inhibited growth of a renal carcinoma tumor model (Renca). In addition, treatment with murine interleukin 2 and interferon γ genes prolonged the survival of Renca tumor-bearing mice and resulted in tumor eradication in 25% of the test animals. Transgene expression was demonstrated in treated tissues by ELISA and immunohistochemical analysis. Significant serum levels of interleukin 6 and interferon γ were detected, demonstrating effective secretion of transgenic proteins from treated skin into the bloodstream. This *in vivo* cytokine gene therapy approach provides a system for evaluating the antitumor properties of various cytokines in different tumor models and has potential utility for human cancer gene therapy.

In situ secretion of certain cytokines by genetically modified tumor or immune system cells can induce inflammatory and/or immune responses which inhibit the growth of certain transplantable tumors. Transgenic cytokines including interleukin 2 (IL-2), IL-4, IL-6, tumor necrosis factor α (TNF α), interferon γ (IFN γ), and granulocyte/macrophage-colony-stimulating factor have been effective in mediating either T-cell-dependent or inflammatory responses leading to tumor regression or rejection (1–6). Mice treated with cytokine-gene-modified tumor cells often rejected subsequent challenges with unmodified tumor cells (4–6). Several clinical trials have been initiated involving use of cytokine-gene-modified autologous tumor cells (7, 8) or tumor-infiltrating lymphocytes (9, 10) for treatment of patients with advanced cancers.

Here we describe an *in vivo* cancer gene therapy approach in which cytokine genes are introduced into tumor-bearing animals by Accell (Agracetus) particle-mediated gene transfer (11–13). This technology utilizes an adjustable electric discharge to generate a shock wave which accelerates DNA-coated gold particles into target cells or tissues, resulting in gene transfer. When compared with other gene delivery methods such as lipofection, calcium phosphate precipitation, or electroporation, the particle-mediated, or “gene gun,” technique can achieve up to 100-fold higher transgene expression levels *ex vivo* or *in vitro* (11, 12). This method has previously allowed highly efficient gene transfer to skin and liver tissues of live animals (13). In this study, we have

developed an *in vivo* gene therapy system by delivering cytokine genes directly into tumor-bearing animals. This *in vivo* and *in situ* gene transfer protocol is designed to bypass the *ex vivo* gene transfer approach commonly employed in previous cancer gene therapy studies.

With this strategy, we investigated several cytokines previously reported to have antitumor effects in defined animal models, including IL-2, IL-6, TNF α and IFN γ . Among a wide array of functions, IL-6 probably serves most importantly as a mediator of inflammation (14). Antitumor activity against several murine tumor cell lines has been described (15). Also, IL-6 gene-transfected tumor cells have been shown to have slower growth or reduced tumorigenicity (16, 17). IFN γ plays a key role in the host response to virus or antigen stimulation and enhances monocyte and natural killer cell function (18). It can exert direct antitumor activity and modulate immune responses by mediating delayed-type hypersensitivity, increasing expression of major histocompatibility complex-encoded antigens on tumor cells, and enhancing tumor cell susceptibility to lysis (18, 19). In addition, IFN γ gene-transfected tumor cells have been shown to exhibit reduced tumorigenicity (20, 21). The antitumor activities of TNF α have also been well documented. Produced by tumor-infiltrating lymphocytes and activated macrophages, it activates cytotoxic T cells and natural killer cells (4). TNF α gene-modified tumor cells have also been demonstrated to have reduced tumorigenicity (22, 23). Although it has potent antitumor activity, systemic administration of TNF α causes severe toxicity (24). As a result, TNF α gene-transfected tumor-infiltrating lymphocytes or tumor cells have been injected to deliver a high local concentration of the cytokine for induction of immune responses (24). IL-2 is known to exert antitumor activity through specific cytotoxic T lymphocytes (25). IL-2 gene-transduced tumors have been demonstrated to attract infiltrating T lymphocytes (26) and can elicit a systemic immune response (27). It is believed that CD8 $^{+}$ cells are the major IL-2-activated effector cells (25), although recent studies have shown that natural killer cells also play a critical role in rejecting IL-2-producing tumors (28).

We report here that efficient *in vivo* transgenic cytokine expression and positive antitumor effects were systematically obtained from human IL-6 (hIL-6), TNF α , murine IFN γ (mIFN γ), and mIL-2 gene treatments. This gene transfer system allows evaluation of the antitumor effects of various cytokines against different tumor types *in vivo* and measurement of the pharmacological kinetics of transgenic cytokine expression. Potential application of this system to gene therapy of human cancers is discussed.

MATERIALS AND METHODS

Cytokine Genes and Expression Plasmids. BCMGIL-6 and BCMGIL-2, containing a hIL-6 or mIL-2 cDNA cloned into a

Abbreviations: IL, interleukin; IFN γ , interferon γ ; TNF α , tumor necrosis factor α ; prefix m, murine; prefix h, human.

†Present address: Department of Dermatology, Children's Memorial Hospital, 2300 Children's Plaza/107, Chicago, IL 60614.

§To whom reprint requests should be addressed.

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BCMG-neo expression vector (17), were kindly provided by Drew Pardoll, Johns Hopkins University, Baltimore, MD. The β -galactosidase gene in pCMV β (Clontech) was replaced with cDNA encoding hTNF α , mTNF α (R & D Systems, Minneapolis), or mIFN γ (kindly provided by G. Opdenakker, Rega Institute, Leuven, Belgium) to create pCMVhTNF, pCMV-mTNF, and pCMVmIFN, respectively. BCMGneo or pCMV-luc (13) was used as the control vector as appropriate.

Tumor Cell Lines and Subcutaneous Tumors. The SP1 methylcholanthrene-induced fibrosarcoma (29) and Renca renal carcinoma cell lines (kindly provided by Robert Wiltrout, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD) were maintained in RPMI 1640 (BioWhittaker) supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 1% minimal Eagle's medium nonessential amino acids, and gentamicin at 50 μ g/ml. Animal use was performed under protocols approved by the Institutional Animal Care and Use Committee. For induction of tumors, 6- to 8-week-old female C57BL/6 or BALB/c mice (Harlan-Sprague-Dawley) were injected subcutaneously on the abdomen with 1×10^5 SP1 or Renca cells, either 1 or 5 days prior to the first DNA treatment.

In Vivo Gene Transfer. Plasmid DNA was purified on Qiagen columns (Qiagen, Chatsworth, CA), precipitated onto gold particles (1–3 μ m) at a density of 2.5 μ g of DNA per mg of particles, and evenly distributed onto a Mylar sheet (1.8 \times 1.8 cm²; 0.1 mg of particles per cm²) as described (30). For cotransfection experiments, two separate cytokine gene plasmid preparations were mixed and coprecipitated onto the gold particles at a dosage of 1.25 μ g of each plasmid per mg of gold particles. Mice were placed on the Accell device (Agracetus) with the target tissues in the path of the particles. Particles were then accelerated by a 20-kV electric discharge, resulting in delivery of the DNA-laden particles to the target tissue.

Cytokine Tumor Gene Therapy Protocol. Established murine tumor cell lines were inoculated subcutaneously into the appropriate mouse host strain on the indicated days prior to the onset of treatment. After the mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg), the surgically exposed tumor site and/or the epidermis overlaying the transplanted tumors was treated *in situ* with test cytokine genes or control DNA. Each treatment with DNA-coated gold particles delivered 0.8 μ g of plasmid DNA per site. In experiments where the tumor was directly exposed to the accelerated particles, a 2-cm incision was made near the tumor site, the subcutaneous tissue was exposed, and test or control plasmid DNA was delivered into the dermis layer at the site of tumor cell injection. The incision was closed with stainless steel clips, and the epidermal tissue overlaying the injected tumor cells was bombarded with the DNA-coated particles. Four to five subsequent epidermal transfections were performed on alternate days in all experiments. In experiments testing the effects of cytokine gene transfer to the epidermis directly over the subcutaneous tumor, particle-mediated gene delivery was performed four or five times at 1- to 2-day intervals as indicated for specific experiments. Antitumor effects were measured 2 weeks (Renca) or 3 weeks (SP1) after the first treatment. The mice were euthanized, the tumors present were excised and weighed, and the average tumor weight in the test and control groups was determined. For survival analysis, mice were sacrificed when tumors reached a predetermined size (≥ 100 mm²), or earlier if the tumor had eroded through the skin.

ELISAs and Immunoperoxidase Staining for Cytokines. Transfected tissues were homogenized and centrifuged, and the supernatants were analyzed by an ELISA specific for hIL-6 (R & D Systems), human TNF α (Biosource, Camarillo, CA), mTNF α (Endogen, Cambridge, MA), or mIFN γ (Genzyme, Boston, MA). Twenty-four hours following gene transfer, tissues at the target sites (1–2 cm²) were excised and fixed for

1 hr with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The tissues were then dehydrated in a graded series of ethanol and embedded in paraffin. Thin (5- μ m) tissue sections were deparaffinized and incubated for 30 min with polyclonal rabbit anti-recombinant hIL-6 or anti-recombinant hTNF α antibodies (Genzyme). To eliminate crossreactivity with mIL-6 or mTNF α , primary antibodies were first incubated overnight in supernatant medium from lipopolysaccharide-stimulated mouse bone marrow cells. Sections were washed with 0.05 M Tris, pH 7.5/10% ethanol/0.25% Tween 20 and incubated with biotinylated goat anti-rabbit IgG for 30 min. After washing, ABC-peroxidase reagent was applied (Vector Laboratories) and avidin-biotin immunocomplexes were visualized with metal-enhanced diaminobenzidine and hydrogen peroxide (Pierce).

RESULTS AND DISCUSSION

Antitumor effects and transgene expression levels of three sets of cytokine genes were evaluated in the present gene therapy study, utilizing a particle-mediated technique for *in vivo* gene transfer. These cytokine genes, including IL-6, IFN γ , TNF α , and IL-2, were initially selected for their reported antitumor effects in specific mouse tumor models and were later found in preliminary screening (data not shown) to confer an apparent antitumor activity. Systematic experiments using these cytokines were then executed, with the results described below.

IL-6. Data from several independent experiments showed that subcutaneous methylcholanthrene-induced fibrosarcoma (SP1) tumors exhibited a reduced growth rate when tumor-bearing mice were treated with hIL-6 DNA, compared with cohorts that received a control DNA vector (Fig. 1A). In initial experiments, both epidermal and dermal tissues surrounding the inoculated tumor were treated. Later experiments showed that epidermal transfections alone resulted in a similar reduction in tumor growth (Fig. 1B), and that the majority of hIL-6 expression resulted from epidermal gene delivery (Fig. 1D). At the discharge voltages used in this study, DNA-coated gold particles effectively transfect epidermal tissues; however, particle penetration to the subcutaneous tumor cells is limited. Thus, these findings suggest that localized expression of hIL-6 by nearby normal tissues plays a key role in the antitumor response. When the epidermal DNA treatments were initiated 5 days after tumor cell inoculation, a similar level of tumor growth reduction was observed (50%), suggesting that *in vivo* transfection with the hIL-6 gene is effective against established tumors.

Histology of the bombarded tissues revealed that the gold particles penetrated three to five cell layers and were primarily located in the skin tissue peripheral to the tumor mass. Immunohistochemistry with an antibody specific for hIL-6 distinctly showed transgenic hIL-6 expression by squamous cells in the epidermal layer (Fig. 2 Upper). To measure the level of transgenic IL-6 production, serum samples, tumors and nearby tissues were collected from *in vivo* transfected mice following euthanization. Two days after transfection, IL-6 levels reached 40 pg/ml in serum (Fig. 1C) and remained at detectable levels (1–5 pg/ml) for up to 7 days as measured by ELISA. The tumor and associated skin tissue extracts contained much higher levels of IL-6, ranging from 200 to 600 pg/ml (Fig. 1D). Thus, high levels of hIL-6 were expressed locally and in serum, compared with the mean physiological concentration of IL-6 in human serum, 1.6 pg/ml (31). The high-level, localized transgene expression apparently creates a cytokine protein gradient from which the transgenic hIL-6 can dissipate into the blood stream via diffusion. Although in the present experiments the mechanism by which IL-6 suppresses tumor growth is not quite clear, both cytotoxic T-cell and inflammatory responses have been previously demonstrated (32).

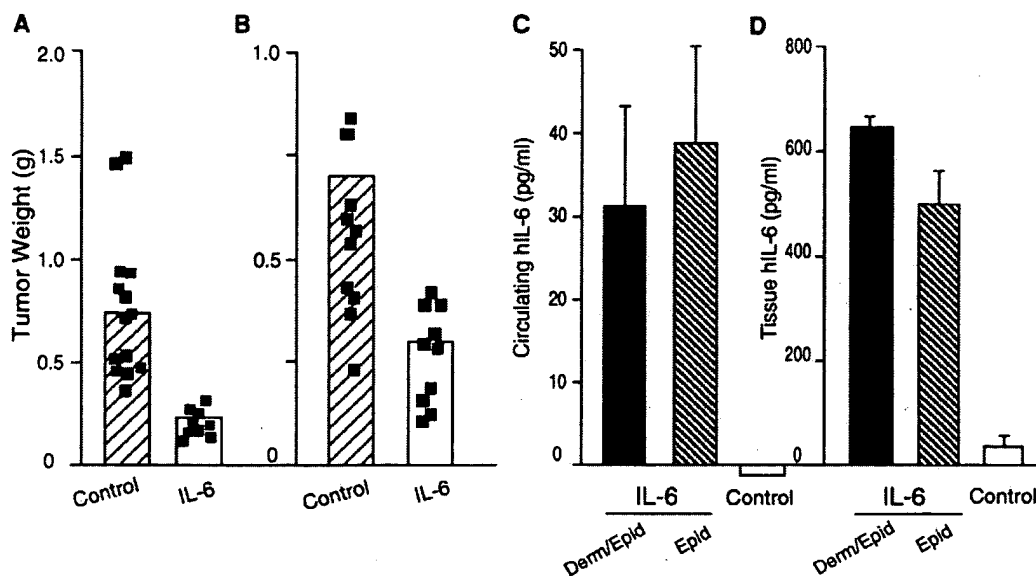


FIG. 1. *In vivo* expression and antitumor effects of a hIL-6 transgene in the mouse fibrosarcoma tumor (SP1) model. (A) Particle-mediated gene transfer of hIL-6 DNA to dermal and epidermal tissues overlaying a subcutaneous tumor results in reduction of tumor growth. The tumor reduction effect from one representative experiment is depicted; two other independent experiments showed similar results. Bars, average tumor weight; filled squares, individual tumor weights. (B) Bombardment of epidermal tissue alone results in similar antitumor effects. (C) Expression of transgenic hIL-6 protein in bombarded mouse skin and tumor tissues determined upon euthanization. Derm, dermis; Epid, epidermis. (D) Detection of transgenic hIL-6 protein in the circulation of test animals. Serum samples were obtained prior to and 1 week after the hIL-6 gene treatment regime was completed.

IFN γ plus TNF α . Cotransfection of IFN γ and TNF α genes *in vivo* by particle bombardment resulted in a drastic reduction of subcutaneous Renca tumor growth (Fig. 3A and B). Three

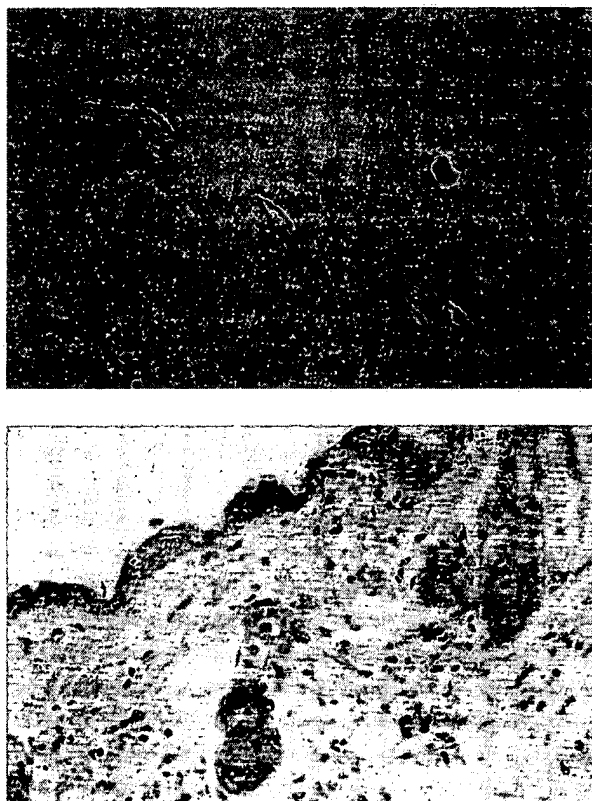


FIG. 2. Immunoperoxidase staining of transgenic hIL-6 (Upper) and hTNF α (Lower) proteins expressed in bombarded skin tissues 24 hr after *in vivo* transfection with the corresponding cDNAs.

experiments, each with 11 or 12 animals per treatment set, showed highly reproducible effects. Compared with controls, average tumor weights excised from mIFN γ /hTNF α -treated animals were reduced by 42%, 48%, and 69% (one-tailed aggregate P value < 0.002). As in the IL-6 experiments, we found that epidermal gene deliveries alone resulted in a reduction of tumor growth (Fig. 2B). Results of three independent mIFN γ /mTNF α experiments showed average tumor weight reductions of 58%, 69%, and 87% (one-tailed aggregate P value ≤ 0.001). Also, mIFN γ /mTNF α gene treatments initiated 5 days rather than 1 day after tumor cell inoculation also resulted in a 35% reduction in average tumor weight (one-tailed P value = 0.06, $n = 10$). Tissue and serum collected for cytokine measurements 24 hr after transfection contained high levels of transgenic IFN γ and TNF α (Fig. 3C and D). Circulating IFN γ levels in the test animals were 186 pg/ml compared with < 1 pg/ml in the controls, which is significantly higher than the normal physiological concentration of IFN γ in human serum, < 10 pg/ml (33).

Immunohistochemical analysis revealed that transgenic hTNF α was produced by the epidermal squamous cells (Fig. 2 Lower). Since the three cytokines studied here showed high expression levels and similar immunohistochemical staining patterns, these studies confirm that particle-mediated gene transfer is a highly efficient technology for expression of transgenic cytokines *in vivo*.

IL-2 plus IFN γ . The effects of mIL-2/IFN γ cytokine gene therapy on the survival of tumor-bearing mice were examined. Renca tumor-bearing mice treated with a combination of murine IL-2 and IFN γ genes had significantly increased survival compared with mice that received a control vector (Fig. 4). In the two experiments shown, 25% of the treated animals remained tumor-free (> 60 days), demonstrating that strong immune and/or inflammatory responses capable of eradicating the tumor were induced.

In this study we evaluated an alternative approach for expression of cytokine transgenes both locally and systemically in tumor-bearing animals. Using two distinct tumor systems, we demonstrated that IL-6 was effective in reducing SP1 fibrosarcoma growth and that both TNF α /IFN γ and IL-2/

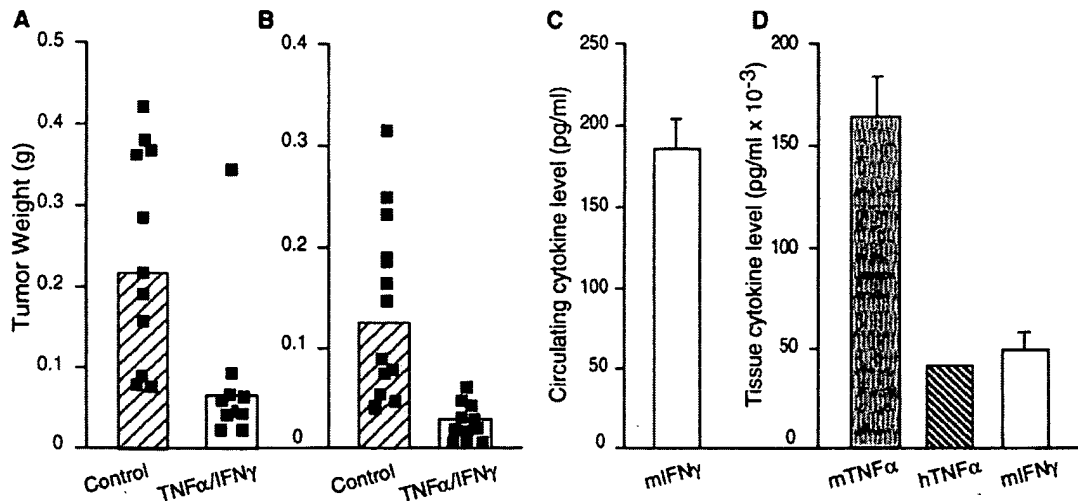


FIG. 3. Antitumor effects mediated by *in vivo* cotransfection of IFN γ and TNF α genes in a mouse Renca tumor model. Average and individual tumor weights measured 14 days after the first gene delivery are depicted. Bars, average tumor weight; filled squares, individual tumor weights. Each set consisted of 12 mice. (A) Tumor reduction effect measured after one dermal and five epidermal treatments with hTNF α and mIFN γ expression plasmids or with control DNA (pCMVluc). Two other independent experiments showed similar results. (B) Increased effectiveness of the murine TNF α gene. In these experiments, mice were treated with five epidermal cotransfections of mTNF α and mIFN γ genes. (C and D) Transgenic cytokine expression in serum and transgenic skin tissues, respectively, collected 24 hr after a single epidermal transfection. ELISAs were performed with commercial kits.

IFN γ gene combinations inhibited Renca tumor growth. In addition, IFN γ /IL-2 gene cotransfection resulted in significantly extended survival of Renca tumor-bearing mice. However, when tested with the current treatment regimen, a murine IL-4 gene construct did not affect the growth of Renca tumors

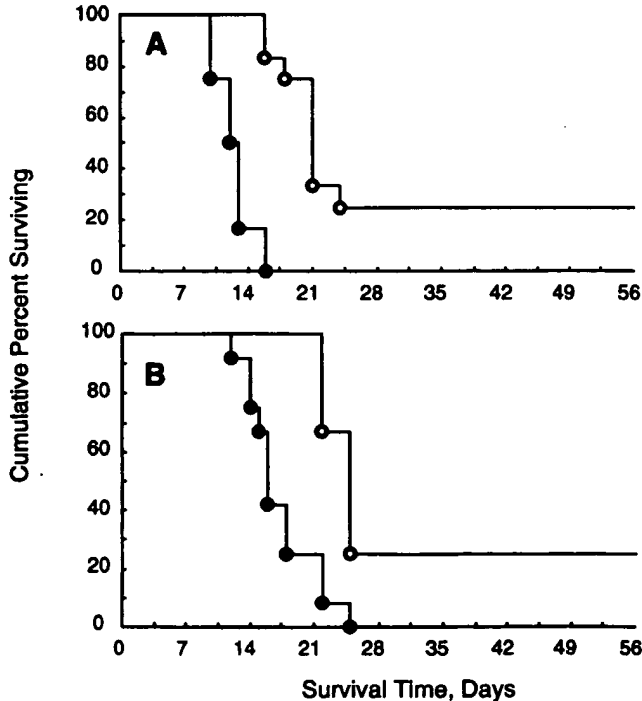


FIG. 4. Kaplan and Meier plots of the cumulative percent survival of Renca tumor-bearing mice bombarded with mIFN γ and mIL-2 genes (○) or pCMVluc as a control (●). Mice were euthanized when the tumor exceeded 1 cm in diameter or erupted through the skin; the interval between tumor cell injection and euthanization is used as the individual survival time. Epidermal bombardments with mIFN γ and mIL-2 genes were as described in Figs. 1 and 3. Two independent experiments (A and B) measuring survival times showed statistically significant differences ($n = 12$, $P \leq 0.005$, Wilcoxon rank-sum test) between treated and control animals.

(data not shown). This indicates that the current *in vivo* gene transfer approach, which results in cytokine secretion in a paracrine fashion, may be effective for certain cytokines but not for others. The efficient *in vivo* production of cytokines that can be achieved by the procedures defined in this study will allow a systematic evaluation of the antitumor activities of additional or newly identified cytokine genes.

In conclusion, this experimental tumor gene therapy system allows cytokine genes to be delivered to specific tissues *in vivo*, creating high peritumoral cytokine concentrations without apparent systemic toxicity. Particle-mediated gene transfer methods are minimally or not dependent on the specific characteristics of the target cell or tissue, since they deliver DNA via a physical force. We therefore suggest that there is potential utility of particle-mediated gene transfer for human cancer gene therapy. Although the underlying mechanisms involved remain to be determined, it is plausible that expression of certain cytokines in the tumor microenvironment mediates a localized or regional immune response which ultimately inhibits tumor growth.

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1. Heaton, K. M. & Grimm, E. A. (1993) *Cancer Immunol. Immunother.* 37, 213–219.
2. Rosenberg, S. A. (1991) *Cancer Res.* 51, 5074–5079.
3. Gutierrez, A. A., Lemoine, N. R. & Sikora, K. (1992) *Lancet* 339, 715–721.
4. Blankenstein, T., Rowley, D. A. & Schreiber, H. (1991) *Curr. Opin. Immunol.* 3, 694–698.
5. Pardoll, D. (1992) *Curr. Opin. Immunol.* 4, 619–623.
6. Colombo, M. P., Modesti, A., Parmiani, G. & Forni, G. (1992) *Cancer Res.* 52, 4853–4857.
7. Rosenberg, S. A. (1992) *J. Am. Med. Assoc.* 17, 2416–2419.
8. Rosenberg, S. A. (1992) *J. Clin. Oncol.* 10, 180–199.
9. Topalian, S. L. & Rosenberg, S. A. (1990) *Important Adv. Oncol.* 19–41.
10. Rosenberg, S. A., Aebbersold, P. M. & Cornetta, K. (1990) *N. Engl. J. Med.* 323, 570–578.
11. Jiao, S., Cheng, L., Wolff, J. A. & Yang, N. S. (1993) *Bio/Technology* 11, 497–502.
12. Yang, N. S., De Luna, C. & Cheng, L. (1994) in *Gene Therapeutics: Methods and Applications of Direct Gene Transfer*, ed. Wolff, J. A. (Birkhäuser, Boston), pp. 193–209.

13. Cheng, L., Ziegelhoffer, P. R. & Yang, N. S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4455-4459.
14. Kishimoto, T. (1992) *Science* **258**, 593-597.
15. Revel, M. (1992) *Res. Immunol.* **143**, 769-773.
16. Mule, J. J., Marcus, S. G., Yang, J. C., Weber, J. S. & Rosenberg, S. A. (1992) *Res. Immunol.* **143**, 777-784.
17. Sun, W. H., Kreisle, R. A., Philips, A. W. & Ershler, W. B. (1992) *Cancer Res.* **52**, 5412-5415.
18. Farrar, M. A. & Schreiber, R. D. (1993) *Annu. Rev. Immunol.* **11**, 571-611.
19. Tuttle, T. M., McCrady, C. W., Inge, T. H., Salour, M. & Bear, H. D. (1993) *Cancer Res.* **53**, 833-839.
20. Watanabe, Y., Kuribayashi, K., Miyatake, S., Hishihara, K., Nakayama, E.-I., Taniyama, T. & Sakata, T.-A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9456-9460.
21. Gansbacher, B., Bannerji, R., Daniels, B., Zier, K., Cronin, K. & Gilboa, E. (1990) *Cancer Res.* **50**, 7820-7825.
22. Asher, A., Mule, J., Kasid, A., Restifo, N., Salo, J., Reichert, C., Jaffe, G., Fendly, B., Kriegler, M. & Rosenberg, S. (1991) *J. Immunol.* **146**, 3227-3234.
23. Blankenstein, T., Qin, Z., Uberla, K., Muller, W., Rosen, H., Volk, H.-D. & Diamantstein, T. (1991) *J. Exp. Med.* **173**, 1047-1052.
24. Cournoy, D. (1993) *Annu. Rev. Immunol.* **11**, 297-329.
25. Swain, S. L. (1991) *Curr. Opin. Immunol.* **3**, 304-310.
26. Fearon, E., Pardoll, D., Itaya, T., Golumbek, P., Levitsky, H., Simons, J., Karasuyama, H., Vogelstein, B. & Frost, P. (1990) *Cell* **60**, 397-403.
27. Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R. & Gilboa, E. (1990) *J. Exp. Med.* **172**, 1217-1224.
28. Porgador, A., Tzehoval, E., Vadai, E., Feldman, M. & Eisenbach, L. (1993) *J. Immunother.* **14**, 191-201.
29. Ho, S. P., Kramer, K. E. & Ershler, W. B. (1990) *Cancer Immunol. Immunother.* **31**, 146-150.
30. Burkholder, J. K., Decker, J. & Yang, N.-S. (1993) *J. Immunol. Methods* **165**, 149-156.
31. R & D Systems (1994) *Cytokine Bull.*, 3-4.
32. Schied, C., Young, R., McDermott, R., Fitzsimmons, L., Scarffe, J. H. & Stern, P. L. (1994) *Cancer Immunol. Immunother.* **38**, 119-126.
33. Melhem M. F., Meisler, A. I., Saito, R., Finley, G. G., Hockman, H. R. & Koski, R. A. (1993) *Blood* **82**, 2038-2044.